Stress-responsiveness and the effect of antidepressants in juvenile animals

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Abstract

Chronic stress is known to be a risk factor for the development of depression and anxiety, disorders which often begin during adolescence. Restraint stress is a commonly used stressor in adult rodents, however the effects of repeated restraint stress in juvenile mice have not been well characterised. Here, I have shown for the first time the behavioural and hormonal effects of repeated restraint stress in both adult and juvenile BALB/c and C57BL/6 mice, as well as the effect of repeated stress on gene expression of components of the HPA axis. Repeated daily restraint stress (2h/day for 3, 7 or 14 days) provoked a robust physiological response evident as increased corticosterone levels and decreased body weight after 14 days (↓8% adults, ↓12% juveniles) compared to controls. Increases in corticosterone after 3 days stress appeared to be greater in juvenile mice (2000% over baseline) than in adults (710% over baseline), in both strains of mice, suggestive of an increased sensitivity of the HPA axis. However, habituation of the stress-response was evident during longer durations of daily exposure to the stressor in both adult and juvenile mice. No changes in gene expression were observed following repeated stress. The behavioural changes seen in response to repeated restraint stress were complex. In juvenile mice, repeated restraint stress evoked an increase in exploratory behaviours in the elevated plus maze, a decrease in time spent immobile in the forced swim test and a decrease in sucrose preference. In adult mice fewer behavioural changes were seen. Interestingly BALB/c and C57BL/6 mice showed qualitatively similar response to 3 days repeated restraint stress despite previous reports that BALB/c mice are more stress-sensitive. The behavioural changes we observed, as a result of prior stress exposure, may represent an adaptive stress-coping response or resilience. Both the hormonal and behavioural effects of stress were more pronounced in juvenile mice than in adults. This wider range of behavioural responses seen in juvenile mice might reflect a greater ability to engage in adaptive stress-coping strategies that likely have beneficial effects evident in future stress challenges.

List of Abbreviations

ACTH	Adrenocorticotrophic hormone
ADAPT	Adolescent depression and psychotherapy trial
ANOVA	Analysis of variance
AVP	Arginine vasopressin
CBT	Cognitive behavioural therapy
cDNA	Complementary deoxyribose nucleic acid
CNS	Central nervous system
Cq	Quantification cycle
CRH	Corticotrophin releasing hormone
CRHR1	Corticotrophin releasing hormone receptor 1
CRHR2	Corticotrophin releasing hormone receptor 2
CSF	Cerebrospinal fluid
DNA	Deoxyribose nucleic acid
DST	Dexamethasone suppression test
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated plus maze
FDA	Food and drug administration
FST	Forced swim test
GR	Glucocorticoid receptor
HPA	Hypothalamic Pituitary Adrenal
i.p.	intraperitoneal
LSD	Least significant difference
MAOI	Monoamine oxidase inhibitor
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid

NACWO	Named animal care and welfare officer
NC3Rs	National centre for the replacement, refinement and reduction of animals in research
NRI	Noradrenaline reuptake inhibitor
NVS	Named veterinary surgeon
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
PGK1	Phosphoglycerate kinase 1
PVN	Paraventricular nucleus
qPCR	Quantitative polymerase chain reaction
RNA	Ribose nucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SNRI	Serotonin and noradrenaline reuptake inhibitor
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitor
TADS	Treatment of adolescents with depression study
TORDIA	Treatment of SSRI-resistant depression in adolescents
V1b	Vasopressin receptor 1b

1 General Introduction

1.1 Depression

Depression is a complex and heterogeneous disorder characterised by a wide variety of psychological, behavioural and physiological disturbances (Cryan et al., 2005a), including low mood, lack of interest or pleasure (anhedonia), inability to concentrate, recurring thoughts of guilt and worthlessness, fatigue, weight changes, alterations in sleep patterns and thoughts of suicide (American Psychiatric Association, 2000). Depression is highly prevalent, with almost 4 million people in the UK suffering from a mood disorder in 2010 (Fineberg et al., 2013). Several distinct clinical subtypes of depression have been suggested, as outlined in Table 1.1. Adolescent depression falls into the early onset depression subtype, which has been defined as a first episode occurring before age 18 years (Baumeister and Parker, 2012).

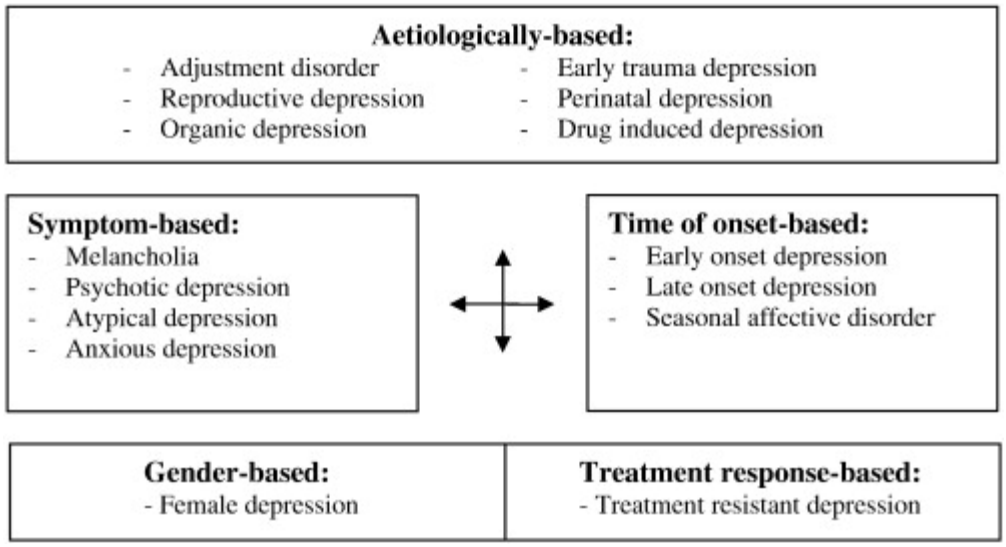

Table 1.1: Frequently proposed subtypes of depression, differentiated by symptoms, aetiology, time of onset, gender and treatment response. Arrows indicate that categories are not necessarily distinct from each other, and patients may fall under more than one category. Figure from Baumeister and Parker (2012).

1.1.1 Adolescent depression

There is increasing evidence that up to half of all adult psychiatric disorders, including depression, have begun by the teenage years (Jones, 2013). It is estimated that depression affects up to up to 6% of adolescents (Masi et al., 2010, Bhatia and Bhatia, 2007, Costello et al., 2006). Depression has a significant impact on relationships with family and friends, performance at school and normal development, and is a leading cause of suicide in this age group (Bhatia and Bhatia, 2007). Adolescent depression tends to recur (Kaufman et al., 2001), and those who experience depression, or subclinical depressive symptoms, during adolescence have a two to three-fold increased risk of depressive illness in adulthood (Jones, 2013, Pine et al., 1999, Dunn and Goodyer, 2006). Therefore, successful treatment of depression in young people is essential.

1.1.2 Aetiology

Stressful life events have been consistently correlated with an increased risk of depression (Kessler, 1997). In adolescents at high-risk of depression, such as those with a parental history of psychiatric disorder, recent stressful life events are associated with the onset of major depression (Goodyer et al., 2000). Similarly, psychosocial adverse events (such as poverty or maltreatment) during young adulthood are predictive of the onset of young adulthood depression (Shanahan et al., 2011). These findings are also observed in adults, when stressful life events significantly increase the risk of a subsequent depressive episode (Kendler et al., 1999). It is known that adolescence is associated with increased experiences of stressful life events, such as problems in family relationships and difficulties at school, compared with childhood (Larson and Ham, 1993). In addition, although both boys and girls experience similar levels of stress during childhood, adolescent girls experience more stressors, and report greater distress to

them, than do adolescent boys, due to the differing nature of their interpersonal relationships (Rudolph, 2002). This correlates with both an increase in the incidence of depression among adolescents compared to children, and with the fact that from adolescence onwards depression is more common in females than males (Guerry and Hastings, 2011).

An increase in circulating cortisol has repeatedly been shown to increase the risk of developing depression. High cortisol, together with higher self-reported depressive symptoms, has been shown to increase the risk of depression in adolescent boys (Owens et al., 2014). In adolescents who are at high-risk of depression, increased cortisol is also associated with the onset of depressive illness (Goodyer et al., 2000). Increased morning salivary cortisol in older adolescents (average age 17.5 years) was associated with increased incidence of depression into adulthood (Ellenbogen et al., 2011). Healthy adolescents with a family history of depression also tend to have elevated evening and nocturnal cortisol (Rao et al., 2009). Given that this elevation in cortisol increases the risk of subsequent depression in both those with and without a family history of depression, it has been suggested that elevated cortisol is a vulnerability marker of depression (Rao et al., 2009). However, it has also been suggested that dysfunction of the hypothalamic pituitary adrenal (HPA) axis occurs as a result of chronic stress, and hence this HPA dysfunction may serve as a mechanism by which stress can lead to the onset of depression (Guerry and Hastings, 2011). This correlates with findings that chronic stress (low socioeconomic status) is associated with hypercortisolaemia in children (Lupien et al., 2000).

1.1.3 Pharmacotherapy

Since fluoxetine (Prozac) was first marketed in 1988, selective serotonin reuptake inhibitors (SSRIs) have become the first-line pharmacological treatment for depression

in both adults and adolescents (Maalouf and Brent, 2010). Whilst all currently available SSRIs are effective in treating depression in adults, response rates are low, with only around 50-60% of patients responding to a first trial of an SSRI, compared with 30% on placebo (Gibbons et al., 2012, Walsh et al., 2002). In adolescents, individual studies of the SSRIs fluoxetine, citalopram, sertraline and paroxetine are inconsistent, with findings both for and against the efficacy of these medications in the treatment of depression (Moreno et al., 2007, Maalouf and Brent, 2010, Masi et al., 2010). A meta-analysis of randomised controlled trials of different SSRIs shows a similar overall response rate of 60% in adolescents, but a higher placebo response rate of almost 50% compared with adults, indicating little benefit of SSRI treatment over placebo (Tsapakis et al., 2008). There is evidence that fluoxetine is more effective than other SSRIs, primarily from the Treatment for Adolescents with Depression Study (TADS) (Maalouf and Brent, 2010, Tsapakis et al., 2008), which revealed that fluoxetine was significantly more effective than placebo in improving depressive symptoms. TADS also showed that fluoxetine in combination with cognitive behavioural therapy (CBT) showed an even greater benefit than fluoxetine alone (March et al., 2004). CBT has also been shown to be beneficial in preventing relapse in adolescents who have previously shown an improvement in response to fluoxetine treatment (Kennard et al., 2014), and a meta-analysis has demonstrated that a combination of antidepressant treatment and psychological therapy is most effective for the treatment of adolescents with treatment-resistant depression (Zhou et al., 2014). However, other studies, such as the Adolescent Depression and Psychotherapy Trial (ADAPT) showed no evidence that the combination of SSRI treatment and CBT resulted in improved outcomes than SSRI treatment alone (Goodyer et al., 2007). Following 6-9 months of treatment, up to 40% of adolescents were still showing depressive symptoms (Kennard et al., 2009, Goodyer et al., 2007), indicating a clear unmet clinical need in the treatment of depression in adolescents.

Older tricyclic antidepressants are also clinically effective in the treatment of depression in adults, although their use in children and adolescents has shown very limited benefit (Hazell et al., 2002, Moreno et al., 2007). The serotonin-noradrenaline reuptake inhibitor (SNRI) venlafaxine, also widely used for the treatment of depression in adults, has demonstrated little or no efficacy in children and adolescents (Emslie et al., 2007, Moreno et al., 2007). Venlafaxine, along with the SSRIs fluoxetine, citalopram and paroxetine, did show efficacy in the TORDIA (Treatment of SSRI-Resistant Depression in Adolescents) study when used as a second treatment in adolescents who showed no improvement to an initial SSRI. 40% of participants showed clinical improvement to a second medication, and there was no difference in the response rate to SSRIs or venlafaxine. A combination of medication and CBT was more effective, with a response rate of 54%. Although there was no difference in the response rate to SSRIs or venlafaxine, the use of venlafaxine was associated with more adverse effects (Brent et al., 2008).

1.1.3.1 Adverse effects of antidepressant use in adolescents

Concerns have been raised over the safety profile of SSRIs, as they have been reported to increase the risk of suicidal thoughts and behaviour, particularly when used in children and adolescents (Masi et al., 2010). Several studies based both in the UK and the USA have reported a modest (1.5-2 fold) but significant increase in the incidence of suicidal ideation and suicide attempts in adolescents, but not adults, treated with antidepressant medication (Hammad et al., 2006, Maalouf and Brent, 2010, Olfson et al., 2006, Bridge et al., 2007). The risk of suicide attempts appears greater in the first week of antidepressant treatment than in the following 3 months (Jick et al., 2004, Simon et al., 2006). However, the risk is highest in the month before the start of treatment, possibly because antidepressant treatment is initiated following a suicide attempt (Simon et al.,

2006). A meta-analysis of published and un-published trials of the SSRIs fluoxetine, paroxetine, sertraline and citalopram, and the SNRI venlafaxine, revealed that only fluoxetine has a favourable risk-benefit profile when used in adolescents. For all other treatments analysed, the increased risk of suicidality outweighed any clinical benefit (Whittington et al., 2004). These findings were followed by a black box warning from the Food and Drug Administration (FDA) in the USA on all antidepressant medications, advising of the risk of increased suicidal behaviour when used in adolescents. Similar warnings from the UK Committee on Safety of Medicines advise against the use of antidepressants in adolescents, with the exception of fluoxetine, where the benefits have been shown to outweigh the risks in this age group (Masi et al., 2010). Consequently, fluoxetine is the only pharmacological treatment currently licensed for the treatment of depressive illness in children and adolescents in the UK (Cousins and Goodyer, 2015), although other SSRIs can be prescribed by specialists.

As deaths by suicide are relatively rare, most data, including the study conducted by the FDA which led directly to the black box warning (Hammad et al., 2006), use suicide attempts as a surrogate marker for an increased risk of suicide. As such, it is not known whether antidepressants increase the risk of completed suicide in adolescents (Olfson et al., 2006). Epidemiological studies examining changes in antidepressant use in adolescents show a clear drop in prescribing following the warnings by both the FDA and the EU regulatory agency (Gibbons et al., 2007, Lu et al., 2014). If the use of antidepressants was associated with an increase in the risk of suicide, this drop in the use of antidepressants would be expected to be associated with a reduction in the rate of suicide among adolescents. However, during this time period where antidepressant use has declined, studies have shown either no change in suicide rate (Lu et al., 2014), or an increase in suicide rate among adolescents (Gibbons et al., 2007). This suggests that the increased risk of untreated depression in adolescents may outweigh the increased risk of antidepressant treatment (Cousins and Goodyer, 2015).

1.2 Stress and the HPA axis

1.2.1 The HPA axis

The hypothalamic-pituitary-adrenal (HPA) axis plays a vital role in regulating the stress response. Stress results in the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus, resulting in the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH enters the circulation and acts on the adrenal glands to cause the synthesis and release of glucocorticoids, predominantly cortisol in humans and corticosterone in rodents, which act via a negative feedback loop to inhibit the further release of CRH, as outlined in Figure 1.1 (Howell and Muglia, 2006, Porter and Gallagher, 2006, Romeo, 2010). Release of cortisol follows a circadian rhythm, with a maximum level shortly after waking, and a minimum at around midnight (Howell and Muglia, 2006, Porter and Gallagher, 2006).

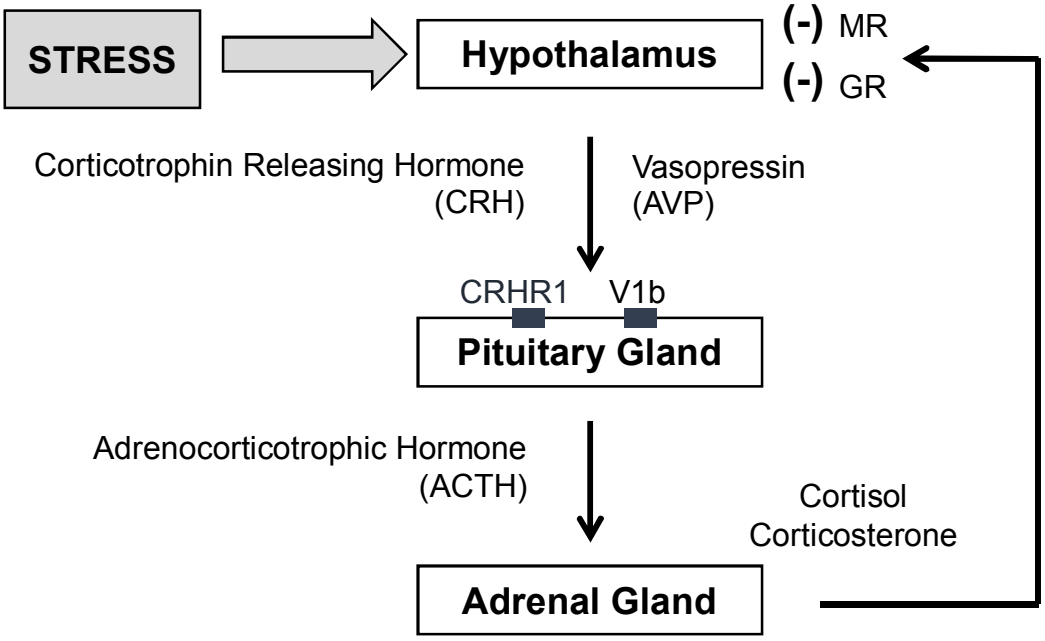

Figure 1.1: The hypothalamic-pituitary-adrenal axis. MR, mineralocorticoid receptor; GR, glucocorticoid receptor; CRHR1, corticotrophin releasing hormone receptor 1; V1b, vasopressin receptor 1b; (-), negative feedback.

CRH is found both in the central nervous system (PVN and amygdala) and in the periphery (gut, skin and adrenal glands). It acts on two different receptors, corticotrophin-releasing hormone receptor 1 (CRHR1) and corticotrophin-releasing hormone receptor 2 (CRHR2), and has a ten-fold higher affinity for CRHR1 than CRHR2 (Bale and Vale, 2004). CRHR1 is more widely distributed in the CNS, and is expressed in the hippocampus, amygdala, pituitary, cortex, cerebellum and olfactory bulb. CRHR2 is expressed in the lateral septum and hypothalamus, but is predominantly expressed in the periphery where it is found in the heart, gastrointestinal tract, lung, skeletal muscle and vasculature (Bale and Vale, 2004). It is CRH activation of CRHR1 in the anterior pituitary which causes the release of ACTH during activation of the HPA axis (Bale and Vale, 2004).

AVP is co-localised with CRH in neurones of the PVN. It only weakly stimulates ACTH release on its own, but acts synergistically with CRH to modulate ACTH release (Aguilera and Rabadan-Diehl, 2000, Scott and Dinan, 2002). Although several different receptor types for AVP exist, it is V1b receptors in the anterior pituitary which are of importance for activation and modulation of the HPA axis (Scott and Dinan, 2002).

Several different glucocorticoids are released on activation of the adrenal cortex, but the most predominant are cortisol in humans, and corticosterone in rodents. Circulating cortisol and corticosterone act on glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) inhibiting release of CRH and hence inhibiting further release of cortisol. This results in a pulsatile secretion pattern of glucocorticoids in response to both stress and circadian variation (Porter and Gallagher, 2006).

GR and MR have been shown to have different roles in the modulation of the stress response through glucocorticoid feedback. MRs have a high affinity for circulating glucocorticoids, are mostly occupied at all stages of the circadian rhythm, and hence are important in modulating basal levels of glucocorticoids. Conversely, GRs have a low affinity for glucocorticoids and so are only occupied when glucocorticoid levels are high. They are therefore important in regulation of the HPA axis when levels of circulating glucocorticoids are high, such as during stress (Howell and Muglia, 2006, Porter and Gallagher, 2006, Scott and Dinan, 2002). Activation of GR and MR during stress results in altered CRH transcription. Raised levels of circulating glucocorticoids reduce transcription of both CRH and CRHR1, resulting in reduced CRHR1 activation and hence a reduction in ACTH release (Scott and Dinan, 2002).

1.2.2 Stress and the HPA axis in adolescence

It has been shown that circadian rhythms undergo development throughout adolescence, in both humans and laboratory mice (Hagenauer et al., 2009). These changes are summarised in Table 1.2.

	Human *(Homo sapiens)*	**Laboratory Mouse *(Mus musculus)***
Magnitude of delay	1-3h	1h?
Sex difference	males > females	only females examined
Rhythms delayed	sleep, melatonin	activity, corticosterone, temperature
No. of experiments	> 20	2
Age of peak delay	15-21 years	unknown, but delay evident at 35-45 days
Age of establishing overt cyclicity in females	menarche: 12-13 years, regular ovulation: 13-16 years	first ovulation: 27-40 days, regular ovulation: 30-80 days
Age of establishing spermatogenesis	12-16 years	n/a
Gonadal dependent	maybe	unknown

Table 1.2: A delay in circadian phase has been observed around the time of puberty in both humans and mice. Table adapted from Hagenauer et al. (2009).

There is a growing body of evidence to suggest that both basal cortisol levels and HPA activity in response to stress increase with both age and sexual maturation during adolescence (Gunnar et al., 2009, Romeo et al., 2016). This is consistent with studies in rats, showing that basal corticosterone increases gradually from 2 weeks of age until adult levels are reached when rats are still adolescent at approximately 6 weeks of age (McCormick and Mathews, 2010). In addition, there are differences in the reaction of the HPA axis to stress in juvenile and adult rats. The increase in corticosterone and ACTH in response to acute stress is more prolonged in prepubertal rats (28 days old) compared

to adults. Conversely, following chronic stress, prepubertal rats initially show a higher increase in ACTH and corticosterone than adults, but these levels return to baseline more quickly (Lui et al., 2012, Romeo et al., 2006). Feedback inhibition of the HPA axis is thought to be reduced in juvenile animals compared to adults, as assessed by a reduction in dexamethasone-induced suppression of corticosterone release (Eiland and Romeo, 2013). However, this is not thought to be related to a reduction in GR or MR expression, which are similar between age groups (Romeo et al., 2013). These differences in HPA reactivity between juvenile and adult animals are thought to be independent of differences in levels of sex hormones between the different age groups (Romeo, 2010). Together, this suggests that adolescence is a sensitive period in terms of the effect of stress exposure on emotional development (Romeo, 2010).

1.2.3 Abnormalities of the HPA axis in depression

Mild to moderate overactivity of the HPA axis has been reported in 30-50% of adult patients with depression (Scott and Dinan, 2002). Elevated cortisol levels in the plasma, the CSF and the urine of depressed patients, along with a flattening of the diurnal variation of cortisol secretion, have been consistently reported in studies over many years (Carroll et al., 1976, Deuschle et al., 1997, Wong et al., 2000). Conversely, in depressed children and adolescents data are conflicting. Baseline cortisol is often normal (Kaufman et al., 1997, Kaufman et al., 2001, Guerry and Hastings, 2011), and any increases in cortisol are generally limited to alterations in diurnal rhythm (a rise in evening cortisol when levels are normally lowest) in a small number of patients (Kaufman et al., 2001). However, a meta-analysis of 17 individual studies revealed that depressed children and adolescents do tend to have significantly higher basal cortisol levels than healthy controls (Lopez-Duran et al., 2009).

Levels of CRH have also been reported to be increased in the cerebral spinal fluid of depressed patients, and in the prefrontal cortex of depressed suicide victims (Merali et al., 2004, Nemeroff et al., 1984). Similarly, depressed patients have increased numbers of CRH-expressing cells in the hypothalamus (Raadsheer et al., 1994), suggesting that increased secretion of CRH may contribute to hyperactivity of the HPA axis in depression. Reports of AVP levels in depressed patients have been conflicting, either showing no change or an elevation, although correlations have been shown between plasma AVP levels and hypercortisolaemia (Scott and Dinan, 2002).

The most commonly used test of HPA function in depression is the dexamethasone suppression test (DST), which gives an indication of the function of the feedback inhibition. Briefly, in the DST, dexamethasone is administered in the late evening and cortisol levels are monitored the following day. In healthy subjects, dexamethasone suppresses cortisol release, but this suppression is not seen in depressed patients (Porter and Gallagher, 2006). Approximately 40-60% of depressed adults and adolescents show this abnormality (Kaufman et al., 2001, Guerry and Hastings, 2011). While rates of cortisol non-suppression reach 50-70% in depressed children, this is thought to be due to the lower dose of dexamethasone used in children (Guerry and Hastings, 2011). It has been suggested that the lack of cortisol suppression is due to reduced responsiveness of the GR in depressed patients (Pariante and Miller, 2001, Baes et al., 2012). Higher levels of non-suppression are often seen in studies of both adult and adolescent patients with increased severity of depressive illness, such as those treated as inpatients, suggesting that the DST may serve as an indicator of clinical severity of depression (Guerry and Hastings, 2011). It has been suggested that the use of a prednisolone suppression test may be more biologically relevant as it is more similar to endogenous glucocorticoids in its ability to bind to both GR and MR, as opposed to dexamethasone which only binds to GR (Pariante et al., 2002). However, as the DST is still widely used in the literature, the DST was used in this thesis.

It is the actions of AVP, rather than CRH, which are thought to contribute to the overactivity of the HPA axis during chronic stress (Aguilera and Rabadan-Diehl, 2000). Studies in both mice and rats using chronic stress paradigms have reported an increase in AVP expression and storage in the hypothalamus, with no increase in CRH expression (Keeney et al., 2006, Scott and Dinan, 2002). Similarly, V1b mRNA is upregulated by high levels of glucocorticoids, contributing to the relative resistance of AVP-mediated ACTH release to glucocorticoid inhibition (Aguilera and Rabadan-Diehl, 2000, Scott and Dinan, 2002). Together, these data suggests that AVP becomes the predominant mediator of the HPA axis during chronic stress.

Overall, hyperactivity of the HPA axis in depressed adults is one of the most consistent biological findings in psychiatry. Similar HPA dysfunction is evident in depressed adolescents, including increased basal cortisol levels and cortisol non-suppression in the DST, and there is evidence that this dysfunction increases with age (Guerry and Hastings, 2011). Furthermore, the difference in HPA function between depressed adolescents and healthy controls appears to be somewhat smaller than in adults, suggesting gradual changes in HPA functioning with age (Guerry and Hastings, 2011).

1.2.4 The effect of treatment of depression on HPA abnormalities

The reduction of elevated cortisol levels in depressed patients on successful treatment of their depressive episode, by both antidepressant drugs and electroconvulsive therapy, has been consistently reported in numerous studies (Gibbons, 1966, Ventura-Junca et al., 2014). Further work has reported that the abnormal DST in depressed patients normalizes on remission of symptoms, particularly in those who remain in remission for at least 6 months (Ising et al., 2007, Ventura-Junca et al., 2014, Zobel et al., 1999), suggesting that relapse is more likely if HPA function doesn't return to normal (Porter and Gallagher, 2006).

It has also been shown that antidepressants increase GR and MR function, by increasing both receptor expression and trafficking (Holsboer, 2000, McQuade and Young, 2000, Pariante and Miller, 2001). There is conflicting evidence of the effect of antidepressants on levels of AVP and CRH, although studies suggest that antidepressant treatment reduces levels of AVP and CRH in the cerebral spinal fluid (Scott and Dinan, 2002).

Together, this suggests that antidepressants may act, in part, by resolving abnormalities in HPA function (Pariante and Miller, 2001, Holsboer, 2000). However, the reasons underlying reduced efficacy of antidepressants in adolescents are not known.

1.3 Stress and the adolescent brain

Adolescence has been shown to be a period of vulnerability for the development of psychiatric disorders, including depression, anxiety, schizophrenia and drug abuse, with up to half of all adult psychiatric disorders beginning by the teenage years (Jones, 2013, Holder and Blaustein, 2014). The experience of stressful events, both psychosocial (such as bullying, social isolation, problems in families and relationships, or death of a close family member) and physical (such as abuse, illness, natural disaster or accident) during adolescence is also associated with an increased risk of developing depression (Holder and Blaustein, 2014, Grant et al., 2004, Eiland and Romeo, 2013). This may in part be attributed to adolescence being a period of marked psychosocial and physiological development (Andersen, 2003, Dahl, 2004). There is also evidence that chronic stress may be more likely to increase the risk of depression than exposure to an acute stressor (McGonagle and Kessler, 1990).

Another important factor that marks adolescence is that it is a period of significant brain maturation in humans and in animal models, which may be linked to the onset of puberty (Spear, 2000, Fuhrmann et al., 2015, Blakemore et al., 2010). Importantly, while absolute

time in rodents and humans are different, in terms of the timeline of development these are somewhat similar, as outlined in Figure 1.2.

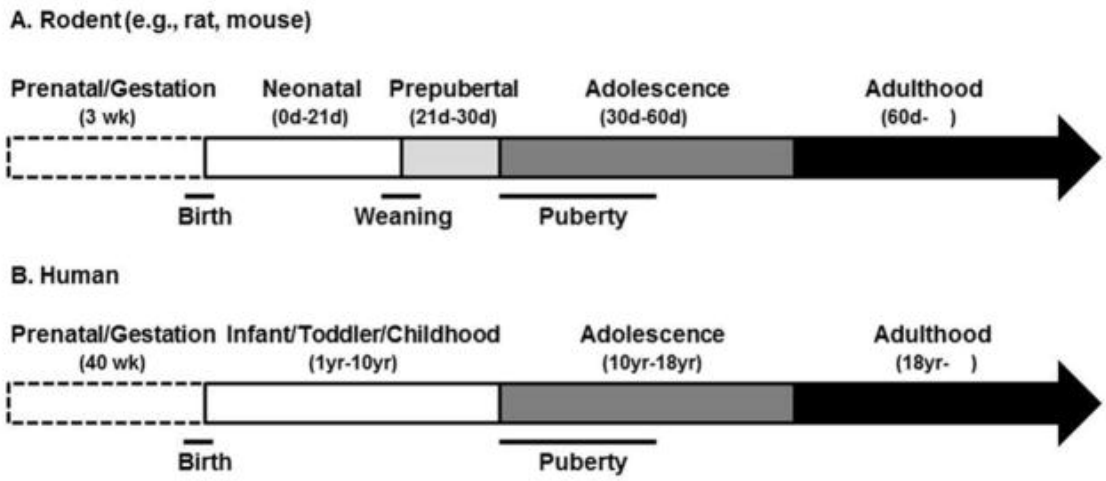

Figure 1.2: Developmental timelines of rodents (A) and humans (B) with approximate age ranges of different stages of development. Abbreviations: d, day; wk, week; yr, year. From Eiland and Romeo (2013).

Of specific relevance to depression, the prefrontal cortex (PFC) and limbic regions undergo significant maturational change. For example, the volume of the PFC declines in adolescence in humans and in rats with the density of spines on pyramidal cells also declining (Spear, 2000). In the PFC, as in other cortical areas, adolescence is also a time of marked synapse elimination particularly of presumed glutamatergic excitatory inputs. Similarly in limbic regions, including the hippocampus there is substantial synaptic pruning evident as a loss of about 25% of NMDA receptors between P28 and P60 in rats (Insel et al., 1990). In addition, significant maturational changes occur in monoaminergic systems during adolescence. For example, forebrain dopaminergic projections to the PFC increase in density during adolescence, but later in adolescence there is a decline in dopamine synthesis and/or turnover in this region (Spear, 2000). Similarly, serotonin

turnover in the nucleus accumbens (but not in the striatum) has been reported to be 4-fold lower in adolescent (P30-P40) rats relative to younger (P10-15) and adult (>P60) animals (Spear, 2000). This complex pattern of brain maturation in a region specific and temporally dynamic manner during adolescence likely contributes to the reported vulnerability for developing psychiatric disorders.

A further factor that contributes to the risk of developing depression is peri-pubertal or adolescent exposure to stressful life experiences (Holder and Blaustein, 2014). However very little is known about how stress impacts on adolescent brain development. Emerging evidence based on studies of adolescent rats indicate that exposure to stressors at prepubertal and early adolescent stages affects cortical and limbic brain regions and that the effects of adolescent stress persist in the adult (Reviewed by Eiland and Romeo, 2013). To understand why antidepressants have reduced efficacy in the adolescent it is important to understand the development of depression in juvenile animals and identify how stress impacts on the brain.

1.4 Animal models of depression

There has been much debate over what criteria are necessary for the evaluation and validation of animal models of depression, particularly in recent years (Nestler and Hyman, 2010, Hendrie and Pickles, 2013, Slattery and Cryan, 2014, Willner and Belzung, 2015). McKinney and Bunney (1969) suggested four minimum requirements for all animal models of depression; 1) the symptoms should be reasonably similar to those in human depression; 2) there should be measurable and clearly defined behavioural changes; 3) the behavioural changes should be reversed by treatments effective in clinical depression; and 4) the results should be reproducible between laboratories. However, more recently, it has been suggested that, given that the main aim of scientific research is to predict what happens in the human disorder, the only

criteria necessary for initially evaluating a model are predictive validity (the ability of the model to predict what happens in the human disorder) and reliability (the consistency with which variables are measured, both within and between laboratories). Other forms of validity include construct validity (whether an animal model measures what it is intended to measure), aetiological validity (similarities between the causes of the response in the animal model and the human disorder) and face validity (similarities between animal behaviour and human symptoms).Whilst these may still be relevant to animal models, they may be considered to be of lesser importance (Geyer and Markou, 1995).

Given the heterogeneity of depression, and that some of the core features of the disorder, such as excessive feelings of guilt and` thoughts of suicide, are impossible to model in rodents, it has been difficult to develop animal models of depression as a whole (Cryan et al., 2002, Yan et al., 2010). In addition, the pathophysiology and aetiology of depression, as well as the complex mechanism of action of clinically useful antidepressant drugs, are still not fully understood, further increasing the difficulty in developing animal models of the entire disorder (Cryan and Mombereau, 2004). Instead, a number of animal models have been developed, which each mimic a specific symptom of the disorder, known as endophenotypes (Cryan et al., 2002, Geyer and Markou, 1995). Animal models of depression have been developed using a combination of genetic approaches, including selective breeding and gene targeting; pharmacological manipulation; environmental exposure to stress; electrical stimulations and brain lesions (Nestler and Hyman, 2010). For example, chronic unpredictable mild stress involves exposing rats or mice to a series of mild physical stressors, such as changes in lighting conditions or temperature, cage tilt, food or water restriction, in an unpredictable manner for several weeks. This procedure has been shown to induce a depression-related phenotype (for example using the sucrose preference test, see Chapter 5.1), which can be reversed by chronic antidepressant treatment (Nestler and Hyman, 2010, Willner et

al., 1987). Social defeat stress involves placing a test mouse into the cage of an unfamiliar, individually housed aggressive mouse, so that the test mouse shows signs of subordination. Often this is followed by sensory exposure of the defeated test mouse to the aggressive resident mouse, without full physical contact, by separating the cage with a perforated divider. Mice undergoing several days of repeated social defeat stress have been shown to develop depression-related behaviours in the forced swim test (see Chapter 5.1), which was reversed by antidepressant treatment (Razzoli et al., 2011). There are also several genetic models of depression, for example the noradrenaline transporter knockout mice and 5-HT_{1A} knockout mice, which show reduced depression-related behaviour in the FST and related tail suspension test (Xu et al., 2000, Czéh et al., 2016).

Alongside these animal models, a number of behavioural paradigms have been developed for assessing depression-related and anxiety-related phenotypes (Lucki, 1997, Cryan et al., 2002, Cryan and Holmes, 2005). These include the forced swim test, sucrose preference test and elevated plus maze (see Chapter 5.1). Although their predictive validity has been increasingly questioned, these behavioural tests have been pharmacologically validated with clinically useful antidepressants and anxiolytics and remain well-established and widely used (Hendrie and Pickles, 2013, Cryan and Sweeney, 2011).

1.5 Hypothesis and Aims

Depression is a mental illness that is increasingly recognized to emerge in adolescence. The available antidepressants have reduced efficacy and increased risks associated with their use in adolescence. Furthermore adolescent depression is characterized by altered HPA activity that is distinct from adult depressed patients. In adults stressful life events lead to HPA activation resulting in hypercortisolaemia and depressive states. In contrast

in adolescence stress is also associated with depression, but with a less robust change in hypercortisol response. In this thesis, I have tested the hypothesis that altered stress-responsiveness in juvenile mice produces a depressive state that is resistant to current monoaminergic antidepressant treatments.

The main aim of this thesis was to develop a model of adolescent depression in juvenile mice. Kim and Han (2006) have shown in adult mice that 2h restraint per day for 14 days induces a pro-depressive behaviour (increased immobility in the forced swim test) and an increase in anxiety-related behaviours (reduced time spent in, and entries into, the open arms of the elevated plus maze). In this thesis, this model was used as a starting point for developing a model of adolescent depression using repeated restraint stress in juvenile mice. In order to assess the effects of repeated restraint stress, I needed to measure corticosterone levels as a measure of HPA function in both adults and juveniles. This necessitated the development of a low stress method for collecting repeated blood samples from juvenile mice with small blood volumes (typically 0.8ml or less) (Chapter 3). I also developed a methodology for assessing mouse welfare while undergoing this protocol to establish humane endpoints in the repeated stress procedure (adapted from Lloyd and Wolfensohn, 1999). The next specific objective was to assess whether or not stress provoked a similar neuroendocrinological response in both adults and juvenile animals. Changes in the function of the HPA axis following repeated stress was also determined using the DST (Chapter 4). Having established that the repeated restraint stress model produced a robust physiological and endocrinological stress response, the next specific objective was to test whether repeated restraint stress in juvenile mice provoked a behavioural change consistent with an increase in depression-related and/or anxiety-related behaviours (Chapter 5). The final objective was to investigate whether repeated stress altered the expression of key components of HPA signalling in juvenile mice, compared to adult mice, which may contribute to altered stress-responsiveness (Chapter 6).

2 General Methods

2.1 Animals

Male BALB/cAnNCrl mice (Charles River, Margate, UK) and male C57BL/6 mice (bred at the University of Bath from a colony originally derived from Charles River) were used throughout these studies. BALB/c and C57BL/6 mice have been shown to differ in their stress responsiveness, with BALB/c mice considered to be more sensitive to the effects of stress than the more stress-resilient C57BL/6 strain (Anisman and Matheson, 2005, Jacobson and Cryan, 2007). Male mice were used as the stress models and behavioural tasks used throughout this thesis are well validated in adult male mice, with experiments extending this to juvenile animals. Pilot studies towards the end of the thesis examined behaviours in female and male mice (see Appendix). Juvenile mice were 4-5 weeks old, and adult mice were 9-10 weeks old at the start of experiments. Mice aged 4-6 weeks can be considered to model human adolescence, as at this age they undergo sexual maturation, have a growth spurt and undergo brain changes markedly similar to adolescent humans (Spear, 2000). C57BL/6 mice were housed in groups of 3-4, and BALB/c mice were housed individually to avoid fighting, in 35 x 20 x 15cm polysulfone cages (Plexx, Elst, The Netherlands) with woodchip bedding (Datesand, Manchester UK) and paper nesting material (Lillico/LBS Biotechnology, Horley, UK). Cages of group housed mice were cleaned weekly, and those of individually housed mice were cleaned fortnightly, following completion of behavioural testing. Mice were maintained on a 12 hour light:dark cycle (lights on at 07:00 h), in a temperature (21 ± 1℃) and humidity (50–60%) controlled environment with food and water available *ad libitum*. All mice were habituated to the animal facility for 4-7 days prior to the start of experiments. Mice were habituated to handling by gentle cupping (Hurst and West, 2010) for 2-3 mins on 1-4 occasions prior to the start of experiments. In all experiments mice were randomly assigned to control or stress treated groups. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986.

2.2 Drugs

All drugs were diluted in 0.9% w/v sodium chloride for injection (saline, Hameln Pharmaceuticals) and administered intraperitoneally (i.p.) in a volume of 10ml/kg. Diazepam (Hameln Pharmaceuticals) was supplied as 2ml vials containing 10mg diazepam, and administered at a dose of 1mg/kg. Fluoxetine hydrochloride (Abcam Biochemicals) was administered at a dose of 10 or 20mg/kg fluoxetine base, where 1.1mg fluoxetine hydrochloride equates to 1mg fluoxetine base. Dexamethasone 21-phosphate disodium (Alfa Aesar) was administered at a dose of 0.1 or 0.01 mg/kg. For acute treatments, drugs were administered 30 minutes prior to behavioural testing. For chronic treatment, drugs were administered once daily between 3-4pm for up to 26 days.

2.3 Stress protocols

2.3.1 Restraint stress

Mice were placed first into a modified 50ml syringe with ventilation holes, which was plugged with the syringe plunger and adjusted depending on the size of the mouse, so that mice were unable to move forwards or backwards. Mice remained in the restraint device for 2 hours (unless otherwise stated), during which time they were monitored constantly. The restraint tubes used in this project are shown in Figure 2.1. Mice underwent restraint stress either once (acute stress), or daily for 3, 7, or 14 consecutive days (repeated stress). For all restraint stress sessions, restraint stress typically occurred from 09:00-11:00 h. The use of restraint stress as a model of depression is discussed in Chapter 4.1.1.

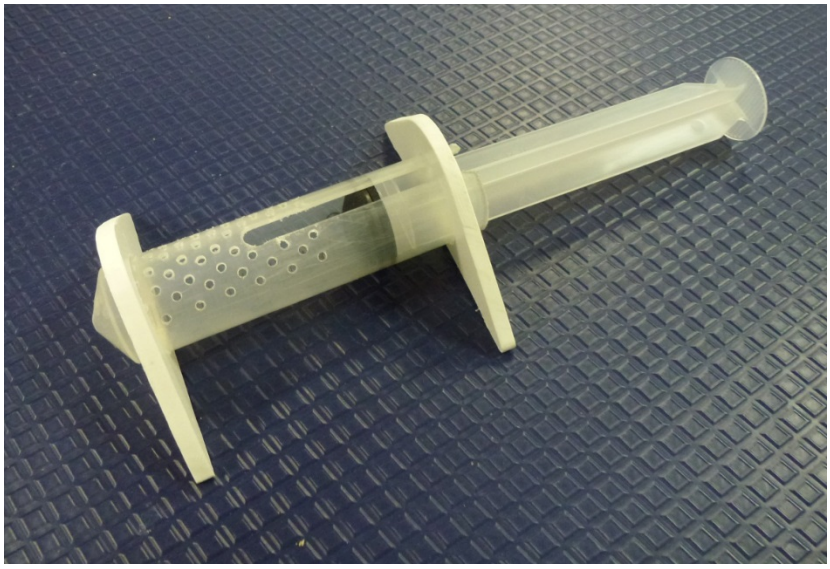

Figure 2.1: Image of a restraint stress tube used throughout this thesis.

2.3.2 Variable stress

A 3 day variable stress protocol was developed from methods previously described (Jacobson-Pick and Richter-Levin, 2010, Brydges et al., 2014). On day 1, mice underwent a 10 min forced swim stress. Stress sessions were conducted in a glass beaker with a diameter of 21cm, height 49cm, filled to a depth of approximately 23cm with water at a temperature of 25 ± 1°C. Following stress, mice were dried with paper towels and placed in a warm holding cage, before being returned to the home cage. The water was replaced, and the beaker cleaned with 70% ethanol, between each mouse. On day 2, mice underwent elevated platform stress. Mice were placed individually in the centre of a small (15cm x15cm) platform, with a 1cm high rim around the edge, elevated 1m above the floor. The platform was a minimum of 55cm from walls or benches in the room. Each mouse had 3 exposures to the elevated platform for 30 min each, with 60 min between each session. The elevated platforms used in the variable stress protocol are shown in Figure 2.2. On day 3, mice underwent 2 hours restraint stress, as described in Chapter 2.3.1. Each of the stressors began at approximately 09:00h.

Figure 2.2: Image of an elevated platform used during the variable stress protocol.

2.3.3 Control, non-stressed mice

Age-matched, sex-matched control mice were used in all experiments. To control for daily handling in repeated stress protocols, control mice were briefly gently handled daily for 1-2 min (09:00-11:00h) by cupping, weighed and returned to their home cage for a similar number of days/duration as the stress protocol required.

2.3.4 Welfare monitoring

For both restraint stress and variable stress protocols, stressed mice were weighed daily and monitored daily for signs of distress using a scoring system (Figure 2.3). This was adapted in conjunction with the named animal care and welfare officer (NACWO) and

named veterinary surgeon (NVS) from Lloyd and Wolfensohn (1999), and monitored mice for changes in bodyweight, appearance and behaviour throughout the stress protocol. Aggregate daily scores were recorded and a score of 0-4 was rated normal. Animals scoring 5-9 required careful monitoring, and scores above 10 required consultation with the NACWO so that relief could be provided. If severe distress was observed the animal could be terminated using a Schedule 1 approved method.

Animal ID:

		Date:		
Body Weight	Daily Body Weight (g)			
	Normal	0		
	Weight Loss <5%	1		
	Weight Loss 5-15%	2		
	Weight Loss >15%	3		
Appearance	Normal	0		
	General Lack of Grooming	1		
	Coat Staring, ocular and nasal discharges	2		
	Piloerection, hunched up	3		
Breathing	Normal Breathing	0		
	Laboured Breathing	3		
Natural Behaviour	Normal	0		
	Minor Changes	1		
	Less mobile and alert, isolated	2		
	Vocalisation, self mutilation, restless or still	3		
Provoked Behaviour	Normal	0		
	Minor depression or exaggerated response	1		
	Moderate change in expected behaviour	2		
	Reacts violently, or very weak and precomatose	3		
Score	If you have scored a 3 more than once, score an extra point for each 3	2-5		
	Total	0-20		

Figure 2.3: Welfare monitoring sheet used throughout all stress studies. Mice were assessed daily to monitor signs of distress. Adapted from Lloyd and Wolfensohn (1999).

3 Validation of a refined technique for taking repeated blood samples from juvenile and adult mice

3.1 Introduction

In order to assess the impact of stress and resulting activation of the HPA axis throughout this thesis, it was necessary to find a suitable method of taking repeated blood samples from both juvenile and adult mice in order to measure changes in corticosterone levels. This presented several challenges when considering blood sampling methodology.

There are several techniques which exist for the collection of blood from mice. Sampling from the tail vein is a commonly used method, although restraint of the mouse is required (such as in a restraint tube), and vasodilation is often necessary in order to insert a needle into the tail vein (Diehl et al., 2001, Fluttert et al., 2000). The saphenous vein is also widely used in mice, which requires shaving of the area and restraint of the mouse. Warming the mouse prior to blood collection may be necessary in order to facilitate blood collection (Hem et al., 1998, Hoff, 2000, Diehl et al., 2001). Tail snip, involving removal of the tip of the tail in order to facilitate bleeding, has also been used as a method of blood collection in mice. However, anaesthesia is recommended, and repeated sampling from the same mouse over a period of time is not possible due to the shortening of the tail (Diehl et al., 2001). Use of the tail snip method without anaesthesia has been found to be stressful, as shown by higher levels of corticosterone in mice following tail bleeding compared with control mice which had only been killed (Tuli et al., 1995).

Collection of repeated blood samples from a single mouse is possible following intravenous cannulation. However, this requires anaesthesia and surgical implantation of the cannula, and animals must be housed individually following surgery (Diehl et al., 2001, Fluttert et al., 2000). These procedures may be stressful to the animal due to the restraint, anaesthesia or warming of the mouse involved (Hoff, 2000, Fluttert et al., 2000). The stressful nature of the blood sampling technique may confound the results of corticosterone analysis, precluding their use when assessing the effects of stress.

Taking repeated blood samples from the same mouse before and after stress allows a significant reduction in the number of mice used over the course of an experiment. Hence a refined minimally invasive method of blood sampling, which allows multiple samples to be taken from the same animal, is desirable (Festing et al., 2002). However, UK Home Office guidelines limit repeated sampling and indicate that no more than 10% of the total blood volume of a mouse may be taken on each occasion, with no more than 25% taken over a 28-day period (NC3Rs, 2015). When working with juvenile mice (4–6 weeks old), which weigh as little as 12g, this limits the volume that can be taken on each occasion to less than 60µl. Therefore blood sampling techniques used in juvenile mice must facilitate the collection of small volumes of blood (up to 40µl), and allow blood flow to be started and stopped easily.

The lateral tail vein is an appropriate route for repeated sampling of small blood volumes from mice without anaesthesia, although vasodilation may be required (Diehl et al., 2001, NC3Rs, 2015). We compared three different blood collection techniques in adult mice before adapting and validating the method developed by Fluttert et al. (2000) as a refined method for repeated blood sampling in juvenile mice.

3.2 Methods

3.2.1 Blood sampling

Preliminary studies aimed to develop a suitable method for taking repeated blood samples from juvenile mice. Three different methods were used initially. To avoid confounding effects of circadian rhythms on corticosterone levels, all blood samples were taken between 11:00h and 13:00h.

3.2.1.1 Warming cabinet method

The warming cabinet method involved heating the mice in a thermostatically-controlled veterinary recovery chamber (Mediheat V1200DT, Peco Services Ltd) for 15 minutes at 38°C, in line with guidelines on the NC3Rs blood sampling website (NC3Rs, 2015) . Mice were then briefly restrained in a restraining tube, a 25G needle was inserted into the lateral tail vein, and the resulting blood droplets collected in heparinised capillary tubes (Hawksley, Sussex, UK). Capillary tubes had a total volume of 80µl, and typically 40µl of blood was collected from each sample.

3.2.1.2 Tail warming method

For the tail warming method, mice were held by hand and the tail was immersed into warm water at 42°C for 20 seconds. A 25G needle was again inserted into the lateral tail vein, and resulting blood droplets were collected in capillary tubes. Again, typically 40µl of blood was collected from each sample.

3.2.1.3 Tail incision method

The tail incision method was developed from the technique established by Fluttert et al. (2000) for use in rats. Mice were held by gentle cupping (Hurst and West, 2010) by one person, while the operator held the tail gently on the bench. The tail was rotated through 90° to expose the lateral tail vein, and a small nick (approximately 2mm wide and 0.5mm deep) was made in the tail with a razor blade, perpendicular to the tail vein, approximately 2 cm from the tip of the tail. Blood droplets were directly collected into capillary tubes. Blood flow was encouraged by gently stroking the tail and in the majority of cases, blood flow stopped spontaneously when stroking was stopped. On occasion it was necessary to apply a small amount of pressure to the tail to stop bleeding. Typically 40µl of blood was collected from each sample.

3.2.2 Corticosterone analysis

In all methods, blood samples were immediately transferred to microcentrifuge tubes containing EDTA (final concentration in sample 3 µg/µl), and stored on ice. Samples were centrifuged at 2000 relative centrifugal force (rcf) for 20 min at 4°C. Plasma (approximately 10-15µl per sample) was removed and stored at -20°C until analysis. The concentration of corticosterone in each plasma sample was determined using a corticosterone enzyme-linked immunosorbent assay (ELISA) (IBL International, Hamburg, Germany). Plasma was diluted 1:10 in Standard 0, and the ELISA was carried out according to the manufacturer's instructions using a reference range of 0-240 nM corticosterone. All samples were measured in duplicate. Briefly, samples and standards were added to a 96-well plate coated with anti-corticosterone antibody, which detects mouse, rat and human corticosterone. Horseradish peroxidase enzyme conjugated to corticosterone was also added to the plate. The plate was thoroughly mixed for 10 seconds, then incubated for 1 hour at room temperature, washed, and enzyme substrate

added. After 15 min incubation, the reaction was halted by the addition of a stop solution and the optical density was read at 450nm using a microplate reader (FLUOstar Optima, BMG Labtech). A standard curve was constructed using a 4 Parameter Logistics curve fit, as calculated by MARS data analysis software (BMG Labtech). Average optical density values from each duplicate sample were determined, and the corticosterone concentration was calculated directly from the standard curve. Intra and inter assay coefficients of variability were reported to be 3.1% and 6.0% respectively.

3.2.3 Statistical analysis

Data were analysed using a repeated measures mixed model analysis (InVivoStat software, version 2.5.0.0) (Clark et al., 2012). Bonferonni's correction was used to adjust for multiple comparisons. Data were $\log_{10}$ transformed prior to analysis to stabilise the variance. All sample sizes are indicated in the figure legends. All data are presented as mean ± SEM, and significance was taken as $P<0.05$.

3.3 Results

Initial studies sought to determine a suitable method of obtaining blood samples. Guidance from the NC3Rs blood sampling website (NC3Rs, 2015) suggested use of either the saphenous vein or lateral tail vein, although pilot studies were unsuccessful in obtaining sufficient blood samples from the saphenous vein. When taking samples from the tail vein, it was found during pilot studies to be necessary to heat the mice, at 38°C for 15 min, to sufficiently dilate the tail vein in order to insert a needle and reliably obtain blood samples (NC3Rs, 2015, Diehl et al., 2001).

To assess the suitability of the use of the tail vein for taking blood as part of a stress protocol, adult and juvenile BALB/c mice had blood samples taken by the warming cabinet method (see Chapter 3.2.1.1). Blood samples were taken at baseline, and immediately following 2h restraint stress. As corticosterone levels reach their peak level approximately 15-30 minutes after the onset of stress, and then fall slowly to baseline levels 60-90 minutes following stress (de Kloet et al., 2005), these post stress blood samples were taken when corticosterone levels were at their peak. 4h following stress, once the peak corticosterone level had fallen, mice were killed by cervical dislocation and a terminal trunk blood sample was taken. 2-way repeated measures mixed model analysis revealed a significant effect of timepoint ($F_{(2,12)}$=312.25, P<0.001), and age*timepoint interaction ($F_{(2,12)}$=8.1, P=0.006) on plasma corticosterone levels (Figure 3.1). While there was a 55% increase in corticosterone immediately following stress in both adult and juvenile mice, this was not statistically significant. Blood samples taken post-mortem following cervical dislocation, 4h after stress, showed corticosterone levels which were 1000% lower than those taken at baseline in adult mice, and 3500% lower in juvenile mice (P<0.001). Thus, the difference between these post-mortem sample values and the high corticosterone levels obtained at baseline showed that taking blood samples using the warming cabinet method was stressful, precluding the use of this method for corticosterone analyses in this project.

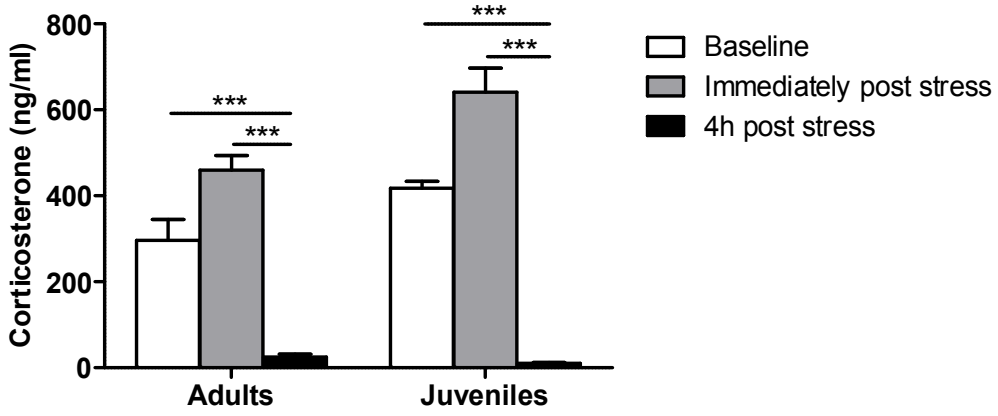

Figure 3.1: Effect of acute restraint stress (2h) on plasma corticosterone in adult and juvenile BALB/c mice. Blood samples were taken from the tail vein using the warming cabinet method at baseline and immediately following restraint. Mice were killed 4h later by cervical dislocation and a terminal blood sample was obtained. Results expressed as mean ± SEM, n=4/group. *P<0.05, ***P<0.001 (post-hoc LSD test).

A comparison was then made between three different methods of taking blood to determine the most refined method, as determined by low basal corticosterone levels. As heating the whole mouse in a warming cabinet, and restraint in a restraint device to obtain a blood sample had proved to be stressful, an alternative method of vasodilation was attempted. The tail warming method (chapter 3.2.1.2) involved warming the tail by immersion in a water bath (42°C for 20s), prior to blood sampling (Parasuraman et al., 2010). This was also compared with the tail incision method (chapter 3.2.1.3), adapted from Fluttert et al. (2000), which did not require dilation of the tail vein.

Adult BALB/c mice had a blood sample taken either by the warming cabinet, tail warming or tail incision method. Mice were then killed 24h later by cervical dislocation and terminal trunk blood samples were obtained. 2-way repeated measures mixed model analysis showed a significant effect of sampling method ($F_{(2,12)}$=7.1, P<0.01), baseline or terminal sample ($F_{(1,12)}$=21.2, P<0.001) and method*sample interaction ($F_{(2,12)}$=10.8, P<0.005) on plasma corticosterone. As found previously, restraining mice and heating in a warming cabinet to take blood was stressful, shown by 300% higher corticosterone levels at baseline compared to a terminal sample 24h later (P<0.001). Corticosterone levels following use of the warming cabinet method were also 220% greater than those in blood taken by the tail warming method (P<0.05), and 350% greater than the tail incision method (P<0.01). For both the tail warming and tail incision methods, corticosterone levels at baseline did not differ from those taken post-mortem following cervical dislocation 24h later (Figure 3.2).

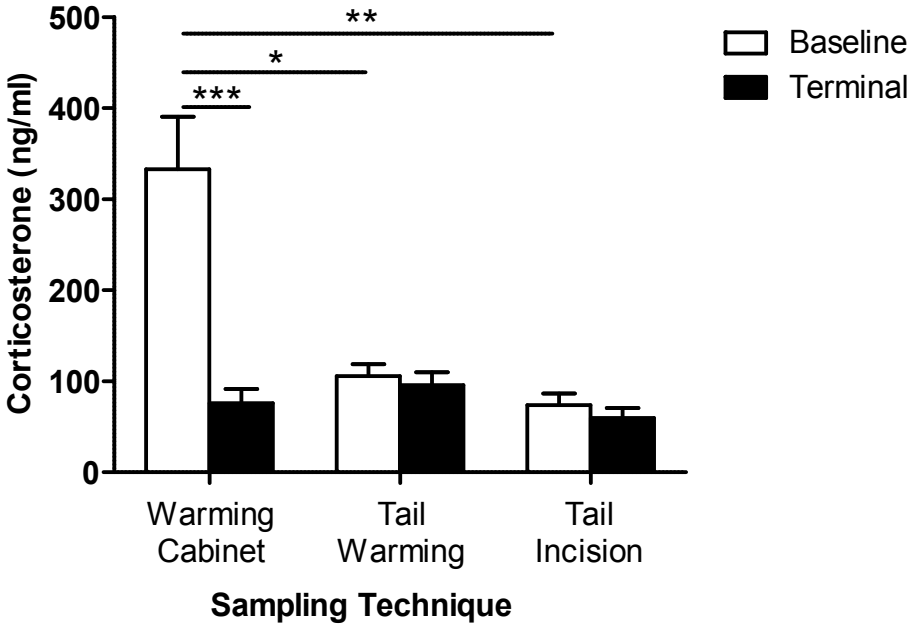

Figure 3.2: Effect of three different blood sampling methods on plasma corticosterone levels in adult BALB/c male mice. 'Baseline' samples were obtained from the lateral tail vein following either warming in a cabinet, or tail warming by immersion in water or tail incision method (no warming). Animals were killed 24 h later by cervical dislocation and 'terminal' samples were obtained. Results are expressed as mean ± SEM, n=5/group. *P<0.05, **P<0.005, ***P<0.001 (post-hoc LSD test).

To confirm the suitability of the tail incision method as a refined method of repeated blood sampling for use in a restraint stress model, the effect of acute restraint stress on corticosterone was determined. Adult and juvenile BALB/c mice had a blood sample taken by the tail incision method at baseline. 24h later, mice were restrained for 2h, with a second blood sample taken immediately following stress. A third sample was taken 24h following restraint stress. 2-way repeated measures mixed model analysis revealed a significant effect of age ($F_{(1,8)}$=13.27, P=0.007) and timepoint ($F_{(2,16)}$=85.58, P<0.001) on corticosterone. At baseline, juvenile mice had 67% lower corticosterone than adults (P<0.05). 2h restraint stress resulted in a 1000% increase in corticosterone in adult mice,

and a 3000% increase in juvenile mice (P<0.001). Corticosterone was no longer significantly different from baseline 24h following stress (Figure 3.3).

On each occasion the total volume of blood collected in the capillary tube was 20-80µl. In a typical experiment, mice had 2 blood samples taken, each of a volume of 40µl. The total volume of blood taken (80µl) was equivalent to 5% of the total blood volume (1.5ml) of a typical adult mouse weighing 25g, and 9% of the total blood volume (0.9ml) of a juvenile mouse weighing 15g.

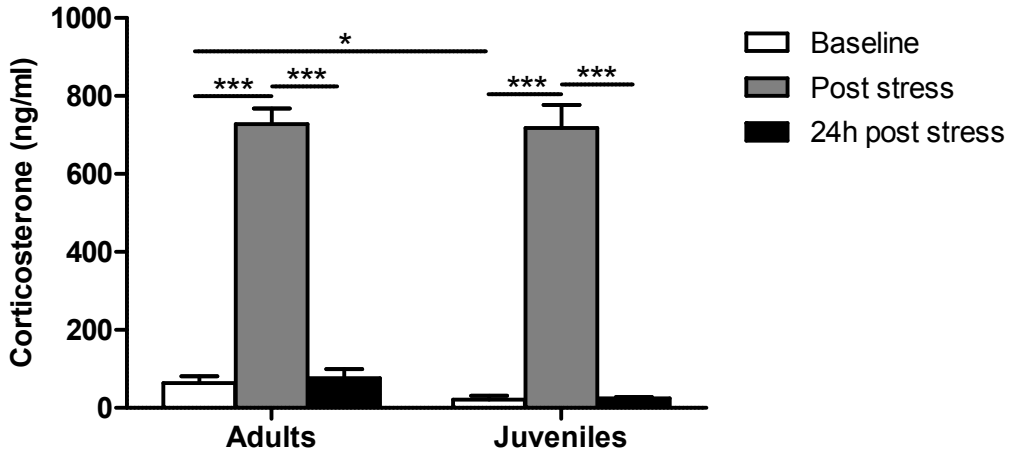

Figure 3.3: Effect of acute restraint stress on plasma corticosterone in adult and juvenile BALB/c mice. Blood samples were taken by the tail incision method at baseline, immediately following 2h restraint stress, and 24h post restraint stress. Results expressed as mean ± SEM, n=5/age. *P<0.05, ***P<0.001 (post-hoc LSD test).

3.4 Discussion

To be suitable for use in studies examining the effects of stress, methods of blood sampling must be refined and minimally invasive to avoid confounding the results of corticosterone analyses. Here, the stressful nature of heating and restraining mice to take blood, as shown by increased corticosterone levels obtained from samples using this method, precluded the use of these techniques for future studies. Conversely, low basal levels of corticosterone were obtained using the tail incision method, indicating that the procedure is not stressful. Furthermore, the levels of corticosterone obtained from the third sample using the tail incision method (24h following stress) are not increased above the baseline samples, demonstrating that repeated sampling is not stressful.

The pattern of corticosterone secretion following stress observed here is similar to that found in other studies. For example, basal levels of corticosterone obtained here (60ng/ml) are similar to those observed by others (<100ng/ml) (Gong et al., 2015, Kim and Han, 2006). Other reports have shown that acute restraint stress for 2h, 8h and 24h has resulted in a significant 250-700% increase in corticosterone above either baseline levels, or above levels in unstressed, control mice (Kim and Han, 2006, Gong et al., 2015, Mizobe et al., 1997).

Bioanalytical techniques have advanced and many assays now require only small sample volumes (microsamples, typically defined as <50µl) to assess drug and chemical exposure in blood, plasma and/or serum (Spreadborough et al., 2013). Microsampling is used in the drug discovery process and in toxicology studies, and a number of methods employing capillary collection have been developed and published in the last two years (Powles-Glover et al., 2014, Caron et al., 2015). The method used here was originally developed for use in adult rats (Fluttert et al., 2000), particularly for use in behavioural studies where implantation of indwelling cannulae is not desirable or methods like tail-cuts are unsuitable for repeated sampling. These studies show that the tail incision or tail nick

method is also suitable for use in adult and juvenile mice. Additionally, mice are not required to be restrained in a restraint device but can be gently cupped throughout the procedure. A further benefit of the tail incision method is that it allows blood flow to be started and stopped easily, facilitating the collection of very small volumes of blood (typically 20–40µl) from juvenile mice. This tail incision method for use in mice has been published as a validated, refined technique for repeated capillary microsampling in juvenile and adult mice (Sadler and Bailey, 2013).

4 Neuroendocrinological effects of repeated stress

4.1 Introduction

4.1.1 Restraint Stress

Restraint stress is straightforward to administer, painless and does not cause bodily harm to the animals (Buynitsky and Mostofsky, 2009). The procedures for restraint stress vary hugely between labs, in terms of method of restraint, duration, frequency, intensity and in the methods and time-points of evaluating the effects of stress (Buynitsky and Mostofsky, 2009). While restraint stress has been widely used in rats (Romeo et al., 2006, Chiba et al., 2012), there are fewer studies using mice. Some attempts have been made to validate different protocols for use in mice, with Kim and Han (2006) finding that 2 or 8 hour daily restraint stress for 14 days is more effective at inducing anxiety and depression-related behaviours in C57BL/6 mice than 6 hours daily for 10 days.

In this thesis, the methodology of Kim and Han (2006) was chosen as it allowed the stress to be delivered to juvenile mice in a time frame where their behaviour could be assessed while they were still juvenile. However, this methodology needed to be validated in terms of its neuroendocrinological effects in juvenile mice as there are no studies where this has been investigated. Both acute and chronic restraint stress have repeatedly been shown to activate the HPA axis, resulting in an increase in plasma corticosterone levels in both rats and mice (Anisman et al., 2001, Gong et al., 2015, Kim and Han, 2006, Romeo et al., 2006). Additionally, monitoring adrenal gland weight has also been used as a marker of stress and persistent HPA activation (Ulrich-Lai et al., 2006).

Disturbance of the HPA axis in depression is evident as impaired glucocorticoid receptor (GR)-mediated negative feedback (see Chapter 1.2.3). This has been verified indirectly, using tests designed to measure the integrity of the mechanism, that include demonstrating non-suppression of cortisol secretion following administration of the synthetic glucocorticoid, dexamethasone (dexamethasone suppression test, DST)

(Porter and Gallagher, 2006). Clinically, the relevance of DST comes from the finding that the response to this test may represent a biomarker of depression, but also a biomarker of treatment success. Indeed, efficacious antidepressant treatment is associated with resolution of the disturbance in the negative feedback in patients who are non-suppressors before treatment, with up to 75% of non-suppressor patients switching to suppressor status coincident with a treatment response (Heuser et al., 1996, Linkowski et al., 1987). Dexamethasone suppression of corticosterone responses has also been demonstrated in mice and rats (Bartolomucci et al., 2004, Moles et al., 2008). In this thesis, we conducted preliminary experiments to establish whether there was any evidence that 3 days repeated restraint stress induced impairments in GR-mediated negative feedback of the HPA axis.

4.1.2 Variable stress

It is known that predictable stressors, such as repeated daily restraint stress, are comparatively less stressful than unpredictable stressors, such as chronic mild unpredictable stress (Anisman and Matheson, 2005). Furthermore, habituation of the corticosterone response is often evident after predicable stress in both mice and rats, demonstrated by lower increases in corticosterone after multiple exposures to the same stressor compared with initial exposures to the stressor (Gong et al., 2015, Kearns and Spencer, 2013). Conversely, unpredictable stress (a different stressor each day) has been shown to result in no significant habituation of the corticosterone response, with corticosterone levels remaining high after each consecutive day of stress in mice (Gong et al., 2015). Unexpectedly increasing the duration of predictable repeated restraint stress has also been shown to increase the corticosterone response compared with rats experiencing the same, predicted, duration of restraint (Kearns and Spencer, 2013). Similarly, altering the physical context in which the predictable stressor is applied can

result in attenuation of the habituation of the corticosterone response, leading to increased corticosterone release in rats (Grissom et al., 2007).

To reduce the possibility of habituation, here I have investigated the effects of variable stress using the paradigm of Tsoory and Richter-Levin (2006), which was developed and shown in juvenile rats. The use of different stressors, such as forced swim stress, exposure to an elevated platform, restraint and footshocks, over 3 consecutive days has repeatedly been used in juvenile mice and rats both to activate the HPA axis, resulting in a stress response, and to induce depression and anxiety-related behaviours Since variable stress protocols elicit stronger stress responses, and reduce the possibility of habituation, than acute or repeated stress protocols, I will use this model developed in pre-pubertal rats and pilot this protocol in juvenile mice.

4.1.3 Chapter aims

In this chapter I have investigated the neuroendocrinological effects of both 3, 7 and 14 days repeated restraint stress, and 3 days variable stress in both adult and juvenile mice. Changes in plasma corticosterone levels following repeated restraint were examined to determine the stressful nature of restraint. This was coupled with analysis of body weight and adrenal gland weight in stressed mice compared with controls. To determine function of negative feedback inhibition of the HPA axis in stressed mice, the effect of repeated restraint stress on suppression of corticosterone release in the DST was also examined.

It has previously been shown that different strains of mouse differ in their response to stress, with BALB/c mice more sensitive to the effects of stress than the C57BL/6 strain (Anisman et al., 2001, Jacobson and Cryan, 2007). This chapter also compares the neuroendocrinological effects of stress in BALB/c mice with the reported more stress resilient C57BL/6 strain.

4.2 Methods

4.2.1 Animals

Adult and juvenile BALB/c and C57BL/6 mice underwent either the restraint stress or variable stress protocol as described in Chapter 2.3. The tail incision method of taking blood was validated as a refined method for repeated blood sampling in adult and juvenile mice (see Chapter 3), and so was used throughout restraint stress and variable stress protocols. Blood samples were taken at baseline, immediately following stress and 24h following stress. Typically 40µl of blood was taken from each sample. A maximum of 10% total blood volume was taken on any one occasion, and no more than 20% blood volume in total from up to 5 samples in adult mice, or 25% blood volume from up to 3 samples in juvenile mice. Baseline blood samples and those following stress were taken at the same time each day (11:00-13:00h) to avoid any confounding effects of circadian variation on corticosterone levels. Blood samples were processed and corticosterone levels in plasma determined by ELISA as previously described (Chapter 3.2.2).

4.2.2 Dexamethasone suppression test (DST)

Function of the HPA axis following stress was also assessed using the DST. Mice had a baseline blood sample taken between 15:00-16:00h the day after the last day of stress. The next day, mice were administered dexamethasone (0.01 or 0.1mg/kg, i.p.) (Bartolomucci et al., 2004) between 09:00-10:00h. Blood samples were taken 6h later, a timepoint commonly used in the DST (Bartolomucci et al., 2004, Groenink et al., 2002), between 15:00-16:00h. Blood samples were processed and corticosterone levels in plasma determined as previously described (Chapter 3.2.2).

4.2.3 Dissection of adrenal glands

To determine any differences in adrenal gland weight between stressed and control mice, mice were killed by cervical dislocation, an incision was made ventrally, adrenal glands were carefully excised and fresh tissue weight was determined.

4.2.4 Statistical analysis

All statistical analysis was performed using InVivoStat software Version 2.5.0.0 (Clark et al., 2012). Corticosterone data were analysed using a repeated measures mixed model analysis with Bonferonni's correction for multiple comparisons, and were $\log_{10}$ transformed prior to analysis to stabilise the variance. Adrenal gland data were analysed using either an unpaired t-test, or 2-way ANOVA as determined by the experimental design. Least significant difference (LSD) test with Bonferonni's correction for multiple comparisons was used for post-hoc comparisons. Bodyweight data were analysed using a repeated measures mixed model analysis, with Bonferonni's correction for multiple comparisons. The relationship between corticosterone and adrenal gland weight was determined using a Pearson's correlation. All sample sizes are indicated in the figure legends. All data are presented as mean ± SEM, and significance was taken as $P<0.05$, as is convention in the literature. However, there has been increasing debate about using $P<0.05$ as an arbitrary cut-off for significance (Dahiru, 2008), and hence in this thesis I have discussed trends in the data where $0.05<P<0.1$.

4.3 Results

4.3.1 Animal welfare monitoring

For both restraint stress and variable stress protocols, stressed mice were weighed daily and monitored daily for signs of distress using the adapted Lloyd and Wolfensohn (1999) scoring system shown in Chapter 2.3.4. Total daily scores for individual mice were typically 1 or 2, with no mouse ever scoring higher than 3.

4.3.2 Neuroendocrinological effects of acute restraint stress

To confirm the stressful nature of restraint stress in adult and juvenile mice, changes in corticosterone were determined following a single session of restraint stress. In adult BALB/c mice, there was a significant effect of stress ($F_{(1,7)}$=14.78, P=0.006), timepoint ($F_{(1,8)}$=32.57, P=0.0005), and stress*timepoint interaction ($F_{(1,8)}$=23.65, P=0.001) on corticosterone (Figure 4.1). In stressed mice, there was a significant 1400% increase in corticosterone above baseline following 2h restraint stress (P<0.001). Similarly, in juvenile mice, there was a significant effect of stress ($F_{(1,7)}$=34.81, P=0.0006), timepoint ($F_{(1,8)}$=10.04, P=0.01) and stress*timepoint interaction ($F_{(1,8)}$=38.34, P=0.0003) on plasma corticosterone (Figure 4.1). 2h restraint stress resulted in a significant 900% increase in corticosterone above baseline (P<0.001).

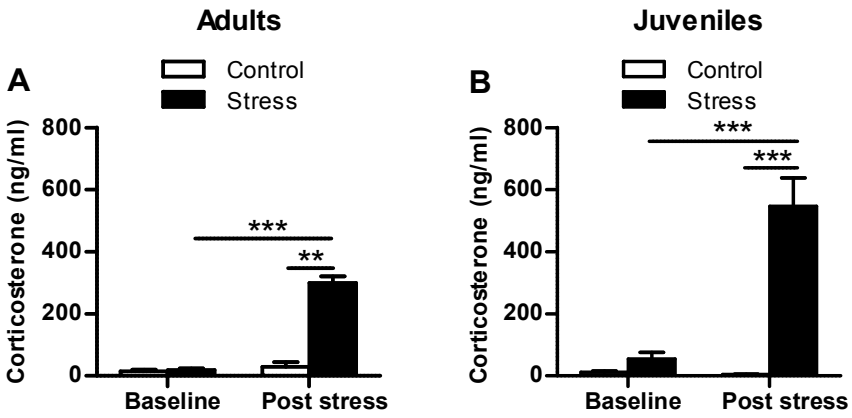

Figure 4.1: Effect of acute restraint stress on plasma corticosterone in (A) adult and (B) juvenile BALB/c mice. Blood samples are taken at baseline and immediately following 2h restraint stress. Results are expressed as mean ± SEM, n=5/group. **P<0.01, ***P<0.001 (post-hoc LSD test).

4.3.3 Neuroendocrinological effects of repeated restraint stress

To determine HPA activation in response to repeated restraint stress, blood samples were taken at baseline and following 3, 7 or 14 days restraint, and plasma levels of corticosterone were determined (Figure 4.2). There were no differences between control and stressed groups in baseline plasma corticosterone measures. Average baseline corticosterone values measured 49ng/ml in adults, and 33ng/ml in juvenile mice. After 3 days restraint stress, there was a significant effect of stress on plasma corticosterone in both adult ($F_{(1,29)}$=49.75, P<0.001) and juvenile BALB/c mice ($F_{(1,27)}$=35.76, P<0.001), with stress resulting in a 710% increase in corticosterone over baseline in adults, and a 2000% increase in juveniles (P<0.001). Similarly, there was a significant effect of 7 days restraint on corticosterone in both adults ($F_{(1,28)}$=18.17, P<0.001) and juveniles ($F_{(1,30)}$=13.9, P<0.001) although this was attenuated compared with 3 days. 14 days restraint significantly increased plasma corticosterone in both adult ($F_{(1,29)}$=69.86, P<0.001) and juvenile mice ($F_{(1,14)}$=10.49, P<0.01), and again these were attenuated compared with 7 days restraint. In adult mice corticosterone was no longer significantly increased over baseline following 14 days restraint (P>0.05).

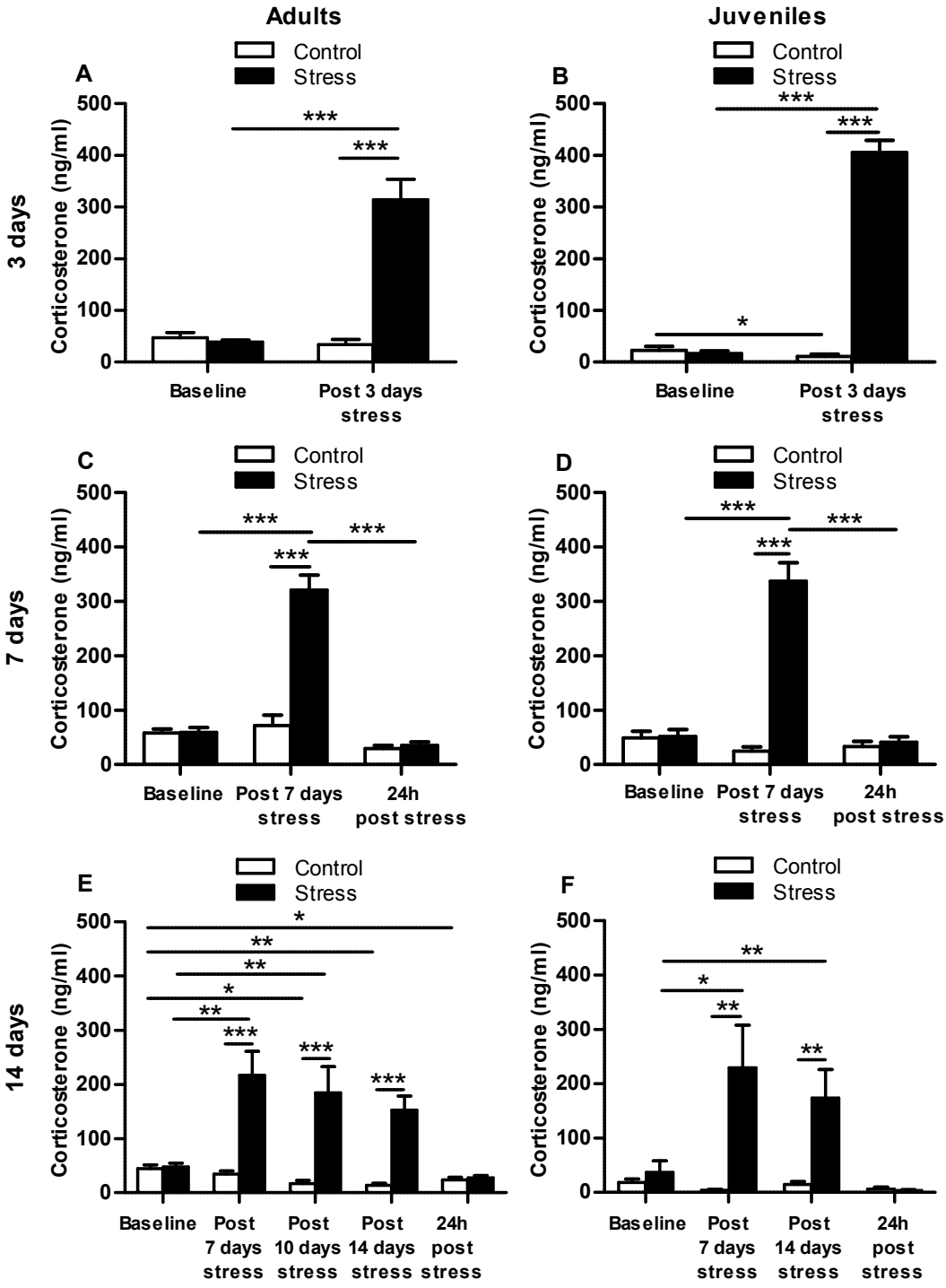

Figure 4.2: Effect of 3, 7 or 14 days restraint stress on plasma corticosterone in adult (A,C,E) and juvenile (B,D,F) BALB/c mice. Blood samples are taken at baseline, immediately following 3, 7 and 14 days restraint, and 24h following the last session of restraint. Results are expressed as mean ± SEM, n=4-16/group. *P<0.05, **P<0.01, ***P<0.001 (post-hoc LSD test).

Adrenal gland weight, corrected for body weight, also demonstrated that the restraint stress procedure impacted on the HPA axis (Figure 4.3). Following 3 days restraint stress, there was a trend towards a significant effect of stress ($F_{(1,25)}$=3.65, P<0.07), and a significant effect of age ($F_{(1,25)}$=12.23, P<0.01) on adrenal gland weight. Stress resulted in a 3% increase in adrenal gland weight in adult mice, and a 10% increase in juvenile mice compared with control, although this was not statistically significant. Extending the duration of the restraint stress to 7 days revealed a significant effect of stress ($F_{(1,26)}$=8.66, P<0.01) and age ($F_{(1,26)}$=19.72, P<0.001) on adrenal gland weight. There was an 8% increase in adrenal gland weight in adult mice (P=0.2), and a 9% increase in juvenile mice (P=0.08), compared with non-stressed controls. Juvenile control mice had significantly heavier adrenal glands than adult control mice (P<0.05). After 14 days restraint, there was a significant effect of stress ($F_{(1,25)}$=8.55, P<0.01) and age ($F_{(1,25)}$=23.93, P<0.0001) on adrenal gland weight. Whilst there was an increase in adrenal gland weight in both stressed adults (9% above control, P=0.2) and juveniles (8% above control, P=0.1), this was not significant following correction for multiple comparisons. Juvenile control mice had significantly heavier adrenal glands than adult control mice (P<0.01).

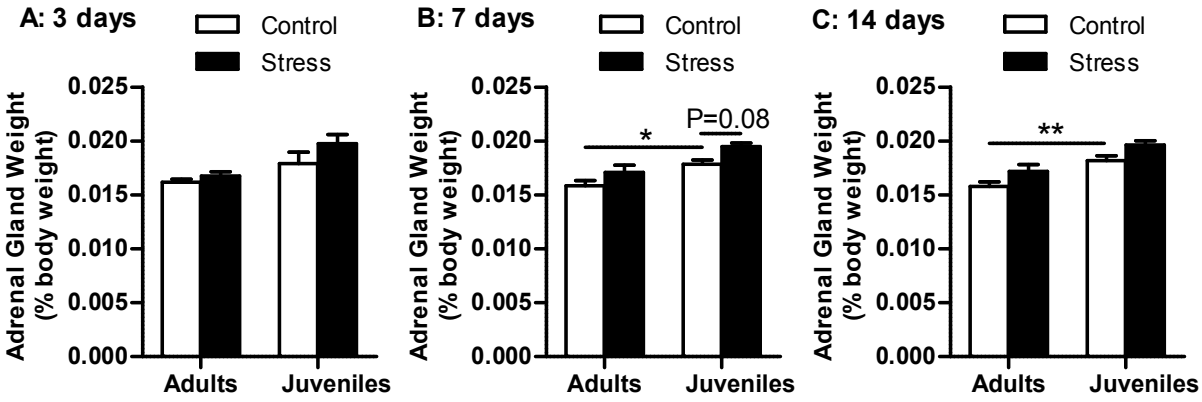

Figure 4.3: Effect of 3, 7 or 14 days restraint stress on adrenal gland weight of adult and juvenile BALB/c mice. Adrenal glands were removed and weighed 2 days after restraint. Results are expressed as mean ± SEM, n=7-8/group. *P<0.05, **P<0.01 (post-hoc LSD test).

A Pearson correlation coefficient was calculated to determine whether there was a relationship between corticosterone levels immediately following stress, and adrenal gland weight in adult and juvenile BALB/c mice. There was a positive correlation between corticosterone and adrenal gland weight in both adult ($r=0.44$, $n=49$, $P<0.005$) and juvenile ($r=0.50$, $n=46$, $P<0.001$) mice (Figure 4.4), although interpretation of this data may be limited due to the group design of the experiments.

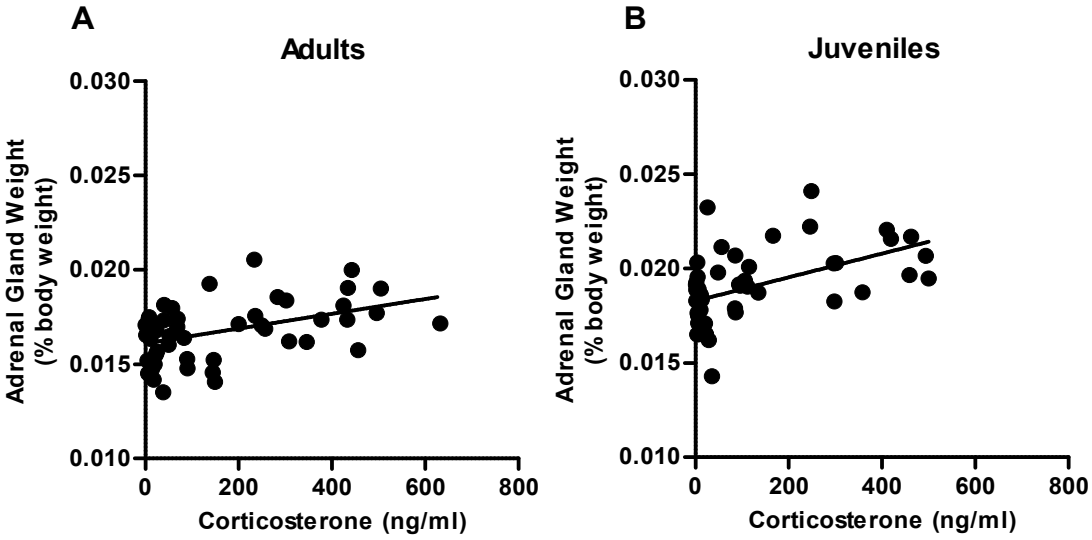

Figure 4.4: Correlation of corticosterone measurement immediately following the last session of restraint/no stress, and adrenal gland weight of adult and juvenile BALB/c mice. Adult mice: $n=49$, $r=0.44$, $P<0.01$. Juvenile mice: $n=46$, $r=0.50$, $P<0.001$ (Pearson's Correlation).

Finally, body weight was recorded daily to assess the effects of repeated restraint stress (Figure 4.5). In BALB/c adult mice, there was a significant effect of stress on bodyweight ($F_{(1,14)}$=36.07, P<0.001), with stressed mice having up to 8% lower bodyweight than controls (P<0.01). Similarly, in juveniles there was a significant effect of stress on bodyweight ($F_{(1,14)}$=8.92, P<0.01), with stressed mice having up to 12% lower bodyweight than non-stressed control mice (P<0.01).

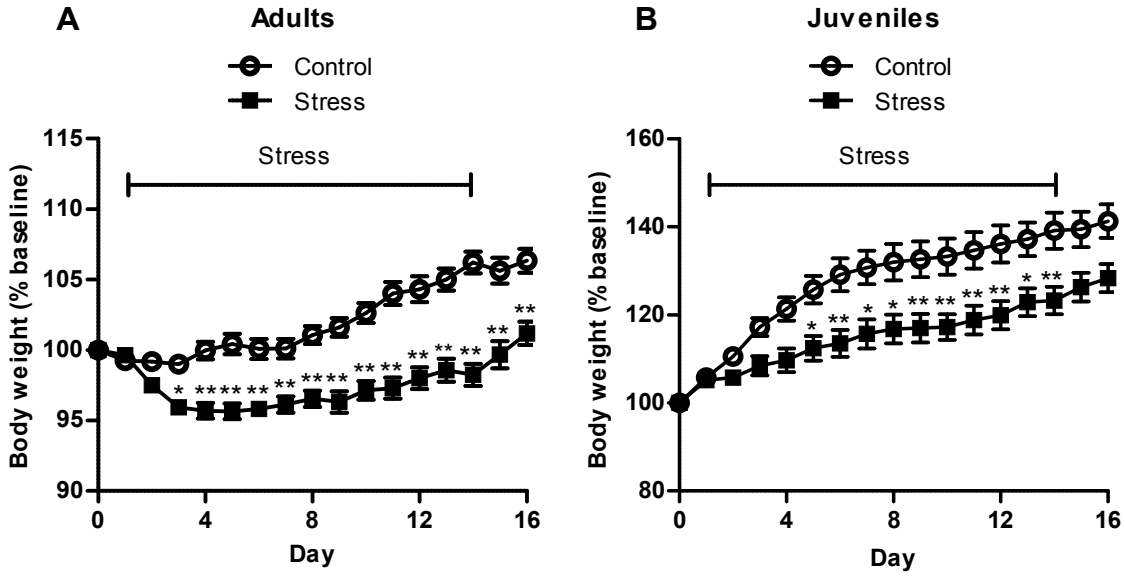

Figure 4.5: Effect of 14 days restraint stress on body weight of (A) adult and (B) juvenile BALB/c mice. Results expressed as mean ± SEM, n=8/group. *P<0.05, **P<0.01 compared with the control group (post-hoc LSD test).

To determine whether a shorter duration of restraint led to less habituation of the HPA response following chronic stress, the effect of 30min restraint for 1 and 3 days on corticosterone was assessed in BALB/c adult mice. Repeated measures mixed model analysis revealed a significant effect of stress ($F_{(1,14)}$=58.2, P<0.001), timepoint ($F_{(2,28)}$=27.81, P<0.001) and stress*timepoint interaction ($F_{(2,28)}$=33.64, P<0.001). Both acute restraint and 3 days repeated restraint resulted in a significant 8-900% increase in corticosterone compared to both baseline (P<0.001) and non-stressed control mice (P<0.001). There was no change in corticosterone in non-stressed control mice (Figure 4.6).

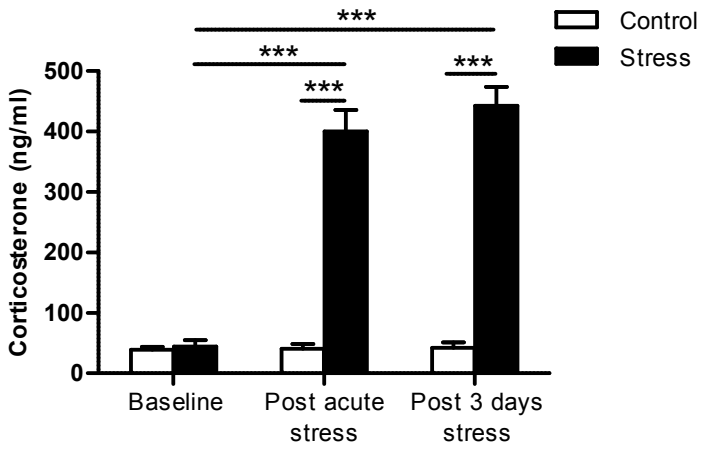

Figure 4.6: Effect of 1 and 3 days restraint stress (30 min/day) on plasma corticosterone in adult BALB/c mice. Blood samples were taken at baseline and immediately following 1 and 3 days restraint stress. Results are expressed as mean ± SEM, n=8/group. ***P<0.001 (post-hoc LSD test).

Chronic stress has been shown to alter function of the HPA axis in mice, resulting in long-lasting changes in the HPA response to future stressors (Brockhurst et al., 2015). To determine any long-term alterations in HPA function following chronic restraint stress, BALB/c adult mice who had previously undergone 14 days restraint stress underwent a single 2h session of restraint, 12-13 days following the end of the original chronic restraint. Corticosterone levels following acute restraint in previously stressed mice were compared with control mice who were undergoing their first session of restraint. No differences were seen in the corticosterone response of previously stressed and control mice following acute restraint (P=0.9, Figure 4.7).

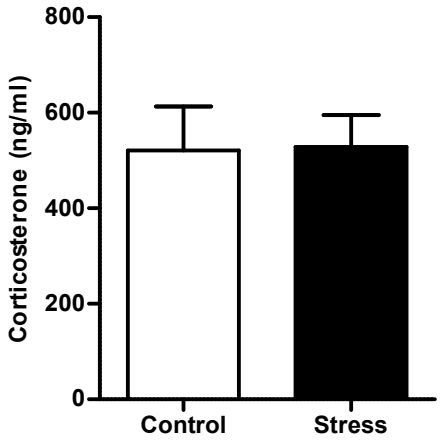

Figure 4.7: Effect of acute restraint stress (2h) on plasma corticosterone in BALB/c adult mice who had previously undergone 14 days restraint stress, compared with control mice with no prior exposure to restraint stress. n=6-7/group.

4.3.4 Neuroendocrinological effects of restraint stress in C57BL/6 mice

BALB/c mice have been reported to be stress sensitive whereas the C57BL/6 strain is relatively stress resilient (Jacobson and Cryan, 2007). The stress responsiveness of C57BL/6 mice was therefore determined in comparison with BALB/c mice.

To determine the effects of acute restraint stress in C57BL/6 mice, blood samples were taken at baseline and immediately following a single 2h session of restraint stress (Figure 4.8). As with BALB/c mice, there was a significant effect of stress ($F_{(1,14)}$=120.16, P<0.001), timepoint ($F_{(1,14)}$=157.26, P<0.001) and stress*timepoint interaction ($F_{(1,14)}$=182.18, P<0.001) on plasma corticosterone. Acute restraint resulted in a significant 330% increase in corticosterone in adult mice (P<0.001), and a smaller 73% increase in corticosterone in juvenile mice (P=0.05).

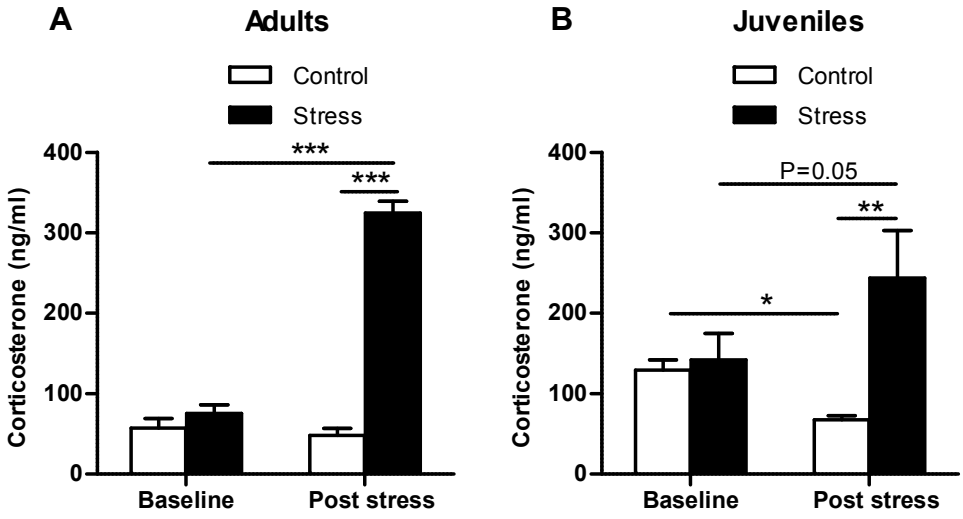

Figure 4.8: Effect of acute restraint stress on plasma corticosterone in (A) adult and (B) juvenile C57BL/6 mice. Blood samples are taken at baseline and immediately following 2h restraint stress. Results are expressed as mean ± SEM, n=8/group (adults), n=5/group (juveniles). *P<0.05, **P<0.01, ***P<0.001 (post-hoc LSD test).

The neuroendocrinological effects of 3 days repeated restraint stress in C57BL/6 adult and juvenile mice were also assessed. There was a significant effect of stress on corticosterone in both adult ($F_{(1,27)}$=109.79, P<0.001) and juvenile ($F_{(1,29)}$=190.51, P<0.001) C57BL/6 mice (Figure 4.9). 3 days restraint stress resulted in a 220% increase in corticosterone over baseline in adult mice, and a 310% increase in juvenile mice (P<0.001). This was accompanied by a 9% increase in adrenal gland weight in adult stressed mice, and a 12% increase in juvenile stressed mice, compared with non-stressed controls (P<0.01, Figure 4.10). Following both acute and 3 days repeated stress in juvenile mice (Figure 4.8B and 4.9B), there is a reduction in corticosterone in the control groups, which may limit the interpretation of the increase in corticosterone in stressed mice. However, in both experiments restraint stress resulted in a significant increase in corticosterone compared with both baseline measures of stressed mice, and corticosterone measures of control mice taken at the same time as post stress values in stressed mice.

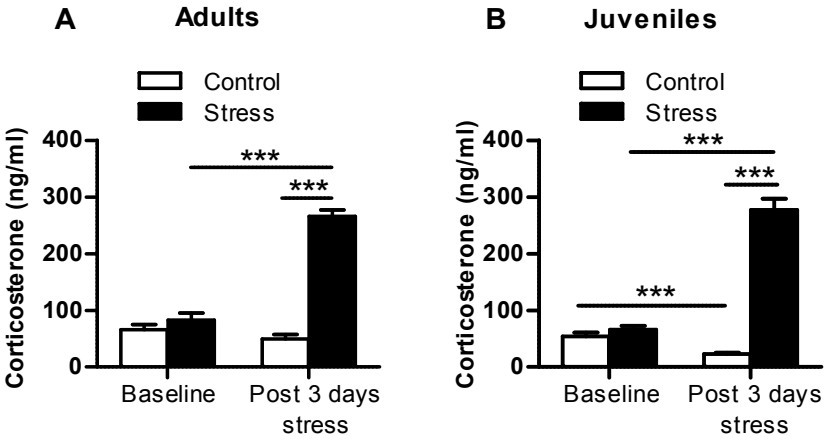

Figure 4.9: Effect of 3 days restraint stress on plasma corticosterone in (A) adult and (B) juvenile C57BL/6 mice. Blood samples were taken at baseline and immediately following 3 days restraint stress. Results are expressed as mean ± SEM, n=15/group. ***P<0.001 (post-hoc LSD test).

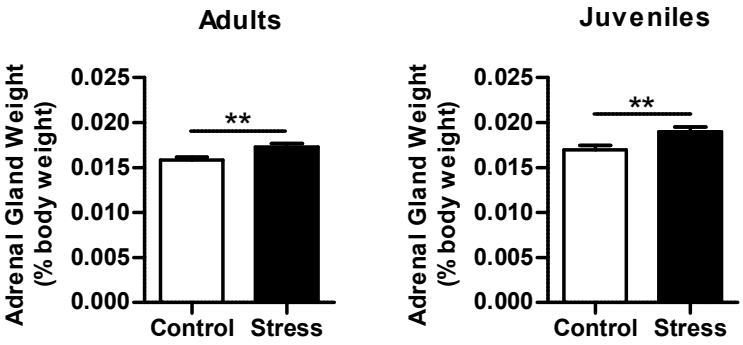

Figure 4.10: Effect of 3 days restraint stress on adrenal gland weight in adult and juvenile C57BL/6 mice. Adrenal glands were removed and weighed the day after the last session of restraint. Results are expressed as mean ± SEM, n=15-21/group. **P<0.01 (unpaired t-test).

Changes in bodyweight were also observed following 3 days restraint stress in C57BL/6 mice (Figure 4.11). The bodyweight of adult mice was 4% lower in stressed mice than in controls following 3 days restraint ($F_{(1,12)}$=15.63, P=0.002). Conversely, in juvenile mice, the reduction in bodyweight in stressed mice was not significant ($F_{(1,14)}$=1.97, P=0.18).

The neuroendocrinological effects of 7 and 14 days restraint stress were not examined in C57BL/6 mice due to time constraints, although future studies would look to examine this.

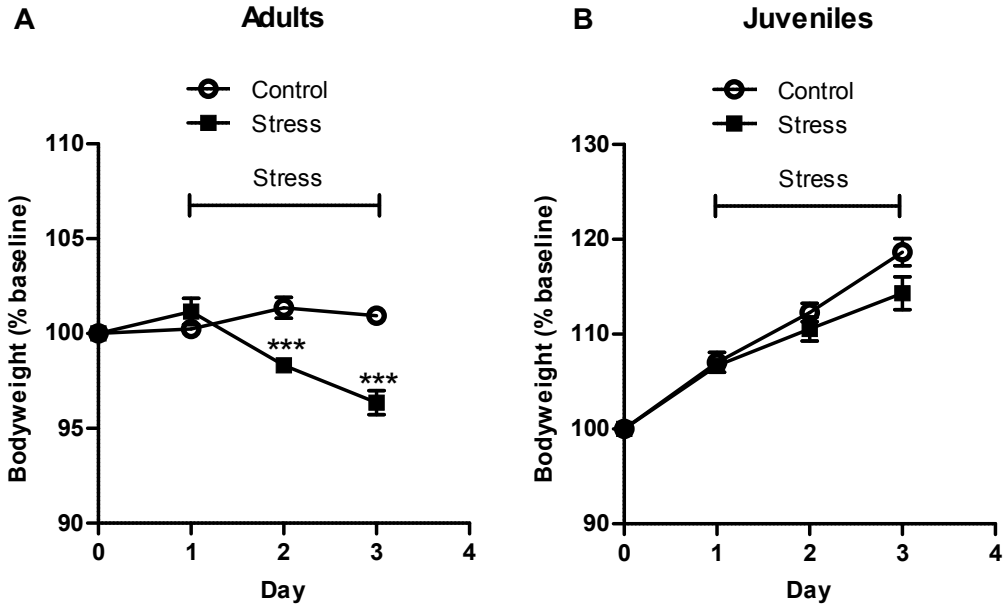

Figure 4.11: Effect of 3 days restraint stress on bodyweight of (A) adult and (B) juvenile C57BL/6 mice. Results expressed as mean ± SEM, n=7-8/group. ***P<0.001 compared with the control group (post-hoc LSD test).

4.3.5 Dexamethasone Suppression Test in BALB/c and C57BL/6 mice

The DST is a commonly used test to assess negative feedback control of HPA function both in depressed patients and in animals following stress (Porter and Gallagher, 2006, Bartolomucci et al., 2004). Initial experiments aimed to determine a suitable dose of dexamethasone to use in the DST (Figure 4.12). There was a significant effect of dose of dexamethasone on suppression of corticosterone release in both BALB/c ($F_{(2,17)}$=8.35, P=0.003) and C57BL/6 ($F_{(2,17)}$=58.03, P<0.0001) mice, in comparison with saline-treated animals. 0.01mg/kg dexamethasone had no effect on corticosterone release in either adult or juvenile, BALB/c or C57BL/6 mice. However, 0.1mg/kg dexamethasone resulted in a significant 80-90% reduction of corticosterone release in BALB/c adult mice, but not juveniles, in this pilot study due to high variability (Figure 4.12A). This variability is likely to be due to a low sample size (n=4/group). Subsequent experiments did show suppression in BALB/c juvenile mice. Suppression of corticosterone release was also seen in C57BL/6 adult and juvenile mice (Figure 4.12B). 0.1mg/kg dexamethasone was the dose used in subsequent DST experiments.

Further experiments confirmed that suppression of corticosterone release by administration of 0.1mg/kg dexamethasone could be observed in comparison to baseline corticosterone values. Adult and juvenile C57BL/6 mice had a blood sample taken at baseline, then the DST was carried out 24h later (Figure 4.12C). Repeated measures mixed model analysis revealed a significant effect of timepoint on corticosterone ($F_{(1,6)}$=43.35, P<0.001), with a significant 90% reduction in corticosterone following dexamethasone administration compared with baseline in both adult and juvenile mice (P<0.01).

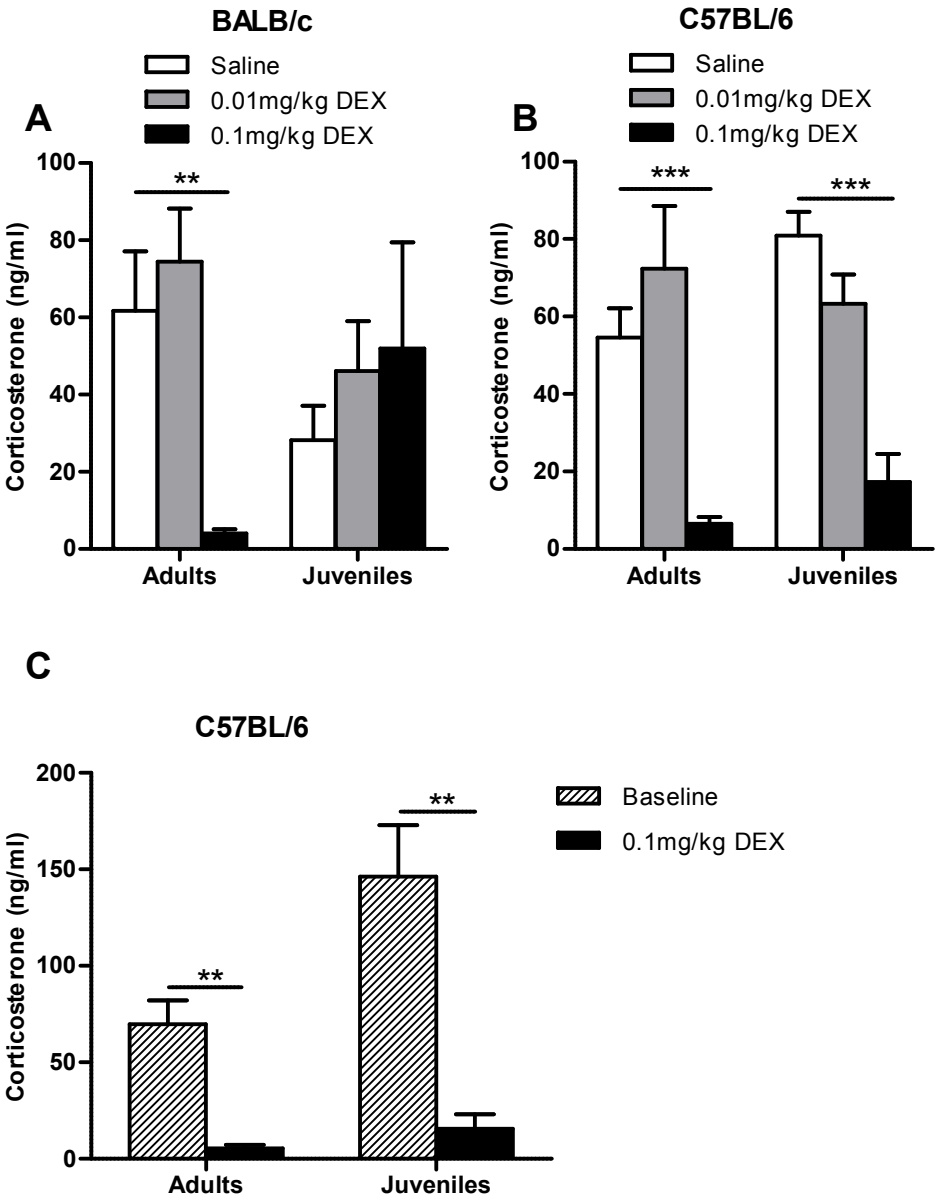

Figure 4.12: DST in adult and juvenile BALB/c (A) and C57BL/6 (B) mice. Blood samples were taken 6h following administration of saline, 0.01mg/kg or 0.1mg/kg dexamethasone (DEX). (C) Blood samples were taken at baseline, and the DST was carried out 24h later, in adult and juvenile C57BL/6 mice. Results expressed as mean ± SEM, n=4/group. **P<0.01, ***P<0.001 compared with either the saline group (A+B), or with baseline (C) (post-hoc LSD test).

To assess changes in the function of negative feedback inhibition of the HPA axis following stress, the effect of 3 days repeated restraint stress on the DST was determined in both BALB/c and C57BL/6 mice (Figure 4.13). In BALB/c adult and juvenile mice, administration of dexamethasone significantly suppressed corticosterone release by around 90% compared with baseline levels ($F_{(1,28)}$=105.05, P<0.001). There was no effect of stress ($F_{(1,27)}$=1.12, P=0.3) or age ($F_{(1,27)}$=0.96, P=0.3) on corticosterone levels following the DST. Similarly, in C57BL/6 mice, dexamethasone resulted in a significant suppression of corticosterone release of around 90% in control mice, and 65% in stressed mice, compared with baseline levels ($F_{(1,28)}$=64.7, P<0.001). Although suppression of corticosterone was lower in stressed mice than controls, this was not significant ($F_{(1,27)}$=2.66, P=0.1).

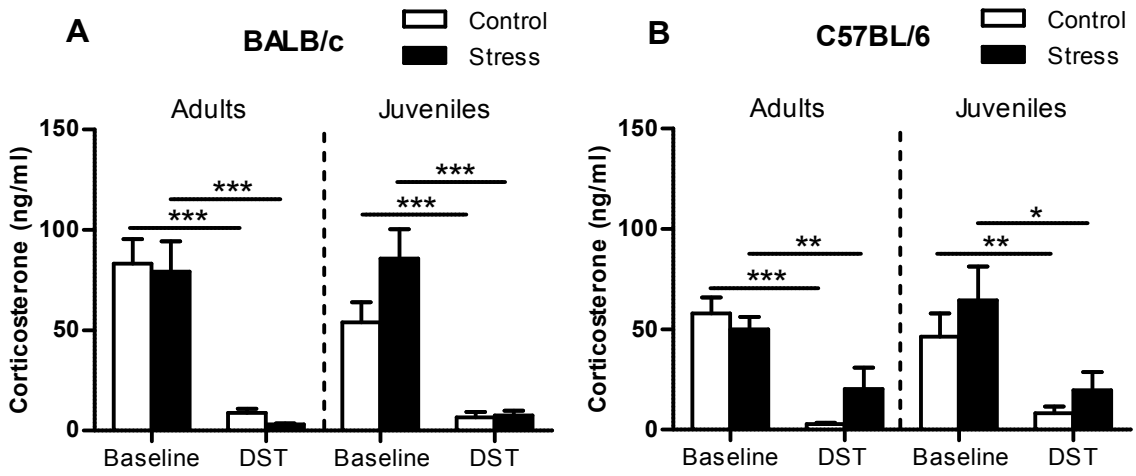

Figure 4.13: Effect of 3 days restraint stress on the DST in adult and juvenile BALB/c (A) and C57BL/6 (B) mice. Mice were restrained daily for 3 days (2h/day). Baseline blood samples were taken on day 4, and the DST occurred on day 5. Results expressed as mean ± SEM, n=8/group. *P<0.05, **P<0.01, ***P<0.001 (post-hoc LSD test).

4.3.6 Neuroendocrinological effects of variable stress

As repeated restraint stress is predictable in nature, the neuroendocrinological effects of 3 days variable stress were investigated in adult and juvenile BALB/c and C57BL/6 mice, to determine whether less habituation of the stress response was evident to an unpredictable variable stress protocol.

Plasma corticosterone levels were assessed in adult mice, immediately following 3 days of variable stress. There was a significant effect of strain ($F_{(1,29)}$=6.18, P=0.019), stress ($F_{(1,29)}$=71.95, P<0.001), timepoint ($F_{(1,31)}$=34.7, P<0.001), strain*stress interaction ($F_{(1,29)}$=8.36, P=0.0072) and stress*test interaction ($F_{(1,31)}$=85.1, P<0.001) on plasma corticosterone (Figure 4.14A). Post-hoc analyses revealed that 3 days variable stress resulted in a significant 1000% increase in corticosterone above baseline in BALB/c mice (P<0.001), and 400% increase in C57BL/6 mice (P<0.001). Although this stress-induced increase in corticosterone was greater in BALB/c mice than C57BL/6 mice, the difference was not statistically significant. There was a trend for C57BL/6 mice to have higher corticosterone at baseline (55ng/ml) than BALB/c mice (31ng/ml) (P<0.1).

Similarly, in juvenile mice, there was a significant effect of strain ($F_{(1,29)}$=29.44, P<0.001), stress ($F_{(1,29)}$=52.64, P<0.001), timepoint ($F_{(1,31)}$=14, P<0.001), stress*timepoint interaction ($F_{(1,31)}$=100.93, P<0.001) and strain*stress*timepoint interaction ($F_{(1,31)}$=9.31, P=0.0046) on plasma corticosterone (Figure 4.14B). 3 days variable stress resulted in a significant 1900% increase in corticosterone above baseline in BALB/c mice (P<0.001), and 400% increase in the C57BL/6 strain (P<0.001). Baseline corticosterone was significantly higher in C57BL/6 mice (101ng/ml) than BALB/c mice (32ng/ml) (P<0.05).

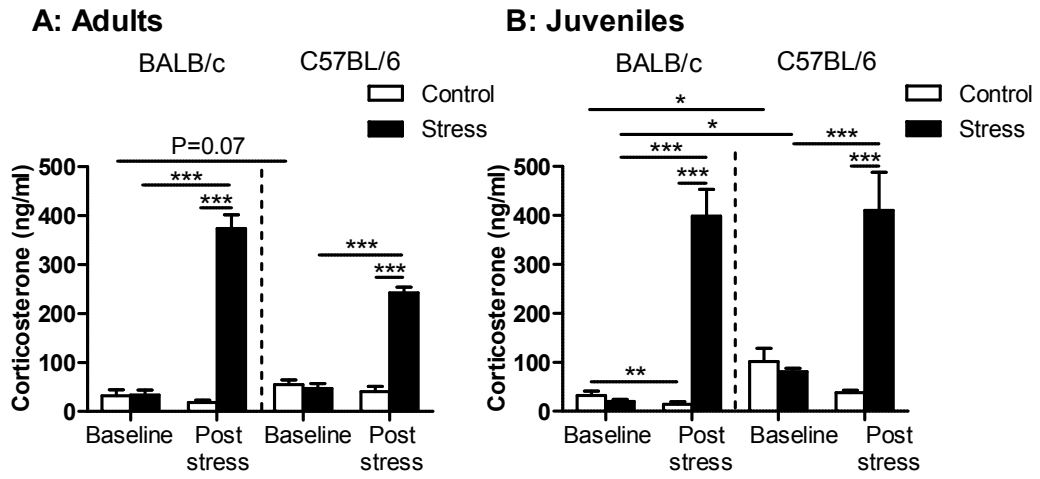

Figure 4.14: Effect of 3 days variable stress on plasma corticosterone in (A) adult and (B) juvenile BALB/c and C57BL/6 mice. Mice had a blood sample taken at baseline and immediately following 3 days variable stress. Results expressed as mean ± SEM, n=8-9/group.***P<0.001, **P<0.01, *P<0.05 (post-hoc LSD test).

The effect of 3 days variable stress on adrenal gland weight was also determined in adult and juvenile BALB/c and C57BL/6 mice (Figure 4.15). In adult mice, there was a significant effect of stress ($F_{(1,27)}$=10.58, P=0.0031). Stress resulted in a significant 10% increase in adrenal gland weight in C57BL/6 mice (P<0.05), whereas in BALB/c mice there was a trend towards a significant 8% increase compared with controls. There was no difference in adrenal gland weight between BALB/c and C57BL/6 mice ($F_{(1,27)}$=1.0, P=0.3) .

Conversely, in juveniles, there was no significant effect of stress on adrenal gland weight ($F_{(1,28)}$=1.7, P=0.2), although there was a significant effect of strain ($F_{(1,28)}$=13.6, P=0.001). C57BL/6 mice had significantly lighter adrenal glands than BALB/c mice (P<0.05).

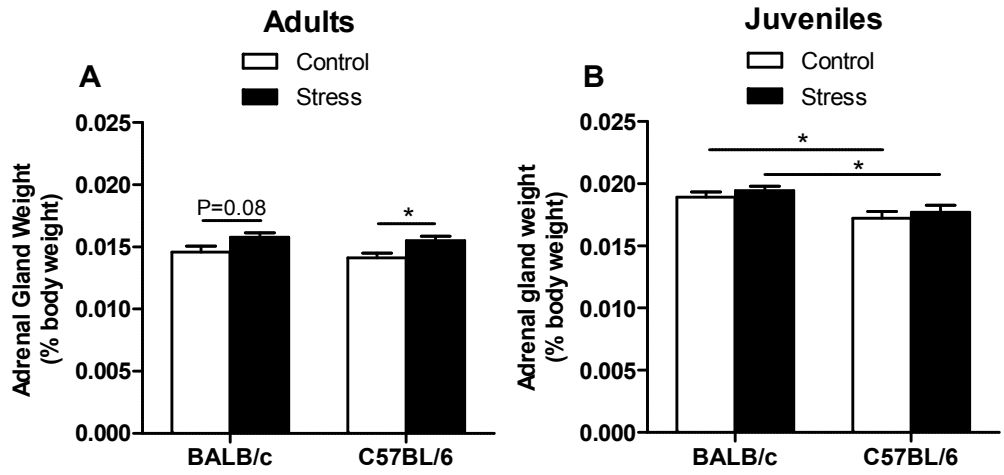

Figure 4.15: Effect of 3 days variable stress on adrenal gland weight in (A) adult and (B) juvenile BALB/c and C57BL/6 mice. Adrenal glands were removed and weighed the day after 3 days variable stress. Results expressed mean ± SEM, n=7-9/group. *P<0.05 (post-hoc LSD test).

A summary of the changes in corticosterone following repeated stress in both BLAB/c and C57BL/6 mice is given in Table 4.1. Similarly, stress-induced changes in adrenal gland weight are summarised in Table 4.2.

	BALB/c		C57BL/6	
	Adult	Juvenile	Adult	Juvenile
3 days restraint stress	↑	↑	↑	↑
7 days restraint stress	↑	↑	-	-
14 days restraint stress	0	↑	-	-
3 days variable stress	↑	↑	↑	↑

Table 4.1: Summary of stress-induced changes in plasma corticosterone following repeated stress. ↑, increase in corticosterone; 0, no effect; -, not tested.

	BALB/c		C57BL/6	
	Adult	Juvenile	Adult	Juvenile
3 days restraint stress	0	0	↑	↑
7 days restraint stress	0	trend to ↑	-	-
14 days restraint stress	0	0	-	-
3 days variable stress	trend to ↑	0	↑	0

Table 4.2: Summary of stress-induced changes in adrenal gland weight following repeated stress. ↑, increase in corticosterone; 0, no effect; -, not tested.

4.4 Discussion

These data show the effects of repeated stress on corticosterone levels, adrenal gland weight, body weight and HPA feedback in both adult and juvenile BALB/c mice and C57BL/6 mice. Different stress paradigms were assessed. Repeated restraint stress of different durations (3, 7 and 14 days) provoked a robust physiological response in BALB/c mice. Plasma corticosterone levels increased and body weight decreased in stressed, compared to control non-stressed, adult and juvenile mice. Interestingly, adrenal gland weight, expressed as a percentage of body weight, was higher in juvenile mice than in adult mice, and showed a trend to increase in stressed, compared to control, adult and juvenile mice.

As already shown in Chapter 3, baseline corticosterone measurements reported here are in line with some values reported in the literature, but may be lower because of the methodology used to collect the blood samples (Sadler and Bailey, 2013). Increases in corticosterone following 3 days stress appeared to be greater in juvenile mice than in adults, in both strains of mice studied, suggestive of the increased sensitivity of the HPA axis seen in juvenile animals (Foilb et al., 2011, Eiland and Romeo, 2013), although this maybe in part because of a lower basal corticosterone level. Previous studies have observed that restraint stress in mice induced increases in corticosterone levels similar to those found in the present study. For example, Romeo et al. (2013) showed a significant increase in corticosterone levels in trunk blood samples from both 30 day old and adult BALB/c and C57BL/6 mice, immediately following a single exposure (30 min) to restraint stress. Corticosterone levels reported were much higher (~700-1200 ng/ml) than the values we obtained following 3 days repeated restraint stress in blood samples collected via the tail nick method (Sadler and Bailey, 2013). Romeo et al. (2013) also showed that restraint stress in juvenile BALB/c mice induced a higher corticosterone response than in adults. However, this data shows a clear attenuation of the corticosterone response in both adult and juvenile BALB/c mice with increasing duration

of restraint stress. Corticosterone was no longer significantly increased above baseline in adult mice following 14 days restraint, indicating habituation to the stressor. This is consistent with previous reports in rats, showing that the HPA axis is less responsive to repeated restraint stress following either 8 or 14 days of restraint, compared to an acute exposure to the stressor (Grissom et al., 2007, Viau and Sawchenko, 2002). 24h following restraint stress, corticosterone levels had returned to normal and were no longer elevated above baseline, indicating that there were no long lasting neuroendocrinological effects of repeated stress.

Substantial evidence indicates that the hormonal stress response, mediated by the HPA axis, can habituate on repeated exposure to stressors. The term “habituation” refers to the reduction in physiological responses elicited by an nth exposure to a repeated homotypic (same) stressor, in comparison to the large responses elicited by acute exposure to that stressor. A change in the context in which the stress is experienced can diminish the magnitude of the habituated HPA response. For example, after exposure to repeated restraint with one of two odours (banana, peppermint), exposure to a matching scent during a test restraint led to lower corticosterone levels compared to those found in animals exposed to the alternate (“switch”) scent during the test restraint (Grissom et al., 2007). On the other hand, exposure to any heterotypic stress (particularly where the stressor differs in modality from the habituated stressor), after HPA habituation to a homotypic stressor, does not elicit cross-stressor habituation but instead facilitation of HPA activity (Armario et al., 1988). In all the repeated restraint stress studies performed here, the context was kept constant to reduce experimental variability. Hence all restraint sessions were at the same time of day, performed in the same room (different to the behavioural room) by the same experimenter. A consequence of this rigorous approach to the experiments probably accounts for the habituation of corticosterone responses after 14 days of a homotypic stressor. In the variable stressor paradigm, where mice were exposed to heterotypic stressors over 3 days, similar plasma corticosterone levels

were observed compared to 3 days repeated restraint stress. This suggests that habituation of the HPA responses must occur over a longer time course than 3 days, and is certainly evident at 14 days.

In addition to assessing corticosterone levels, experiments were also conducted to assess whether repeated restraint stress altered GR mediated negative feedback of the HPA axis. In both BALB/c and C57BL/6 mice, adults and juveniles, showed significant suppression of corticosterone responses following administration of 0.1 mg/kg dexamethasone. There was no difference between control and stressed animals. In these studies the impact of 3 days repeated restraint stress on DST was investigated. It would be anticipated that significant impairment of GR mediated negative feedback would take time to develop. In future studies, it would be of interest to examine dexamethasone suppression in animals exposed to 7 -14 days repeated restraint stress.

These studies have compared the effects of restraint stress on two different strains of mice. There have been several studies looking at strain differences in stress responsiveness, with BALB/c mice considered to be more sensitive to the effects of stress than the more stress-resilient C57BL/6 strain (Anisman and Matheson, 2005, Jacobson and Cryan, 2007). Consistent with this, our results show an increased corticosterone response to 3 days restraint stress in BALB/c mice (700% increase above baseline) compared with C57BL/6 mice (220% increase above baseline). Similarly, 3 days variable stress resulted in a 1000% increase in corticosterone above baseline in BALB/c adult mice, and a 400% increase in C57BL/6 adult mice. Again, in juvenile mice, 3 days variable stress resulted in a greater increase in corticosterone in BALB/c mice (1900% above baseline) than in C57BL/6 mice (400% above baseline). Although these stress-induced increases in corticosterone were greater in BALB/c mice than C57BL/6 mice, the differences were not statistically significant.

In conclusion, repeated restraint stress induced a significant increase in plasma corticosterone responses compared to control mice at 3, 7, 10 and 14 days of repeated stress. Responses at 10 and 14 days were attenuated compared to 3 and 7 days suggesting that habituation to a homotypic stressor was occurring. Juvenile mice tended to have a higher % change than in adults, in part because of a lower basal corticosterone level. The restraint stress procedure also impacted on adrenal gland weight which increased and correlated with plasma corticosterone levels. Taken together, these data indicate that the restraint stress procedures used produced a robust neuroendocrinological stress response.

5 Behavioural effects of repeated stress

5.1 Introduction

A variety of behavioural paradigms have been developed to assess different aspects of depression-related behaviour. The particular tests chosen to assess behaviours here are commonly used, pharmacologically validated tasks in mice. While these tests have been well validated for use in adult mice, it is less clear how well they have been validated for use in juvenile mice.

5.1.1 Forced swim test

The forced swim test (FST) was first developed by Porsolt et al. (1978) for use in rats, and subsequently modified for use in mice. The test involves placing the animal into a beaker of deep water for 6 minutes, and recording the amount of time it spends swimming, climbing and immobile as well as the latency to the first period of immobility. An antidepressant effect is defined as a decrease in the amount of time spent immobile, resulting in a corresponding increase in climbing and swimming behaviours. Different classes of clinically effective antidepressants have shown to have antidepressant effects in the FST in mice (Cryan and Holmes, 2005), demonstrating the pharmacological validity of the test. These include the SSRIs fluoxetine, paroxetine and citalopram (Cryan et al., 2005b, David et al., 2003, Lucki et al., 2001), the tricyclic antidepressants imipramine and desipramine (David et al., 2003, Lucki et al., 2001), the monoamine oxidase inhibitor (MAOI) moclobemide (Cryan et al., 2005b), the noradrenaline reuptake inhibitor (NRI) reboxetine (Cryan et al., 2005b), as well as the atypical antidepressants ketamine (Autry et al., 2011) and bupropion (David et al., 2003).

Different strains of mice have been shown to differ in both their behaviour in the FST, as well as their response to antidepressant treatment (Jacobson and Cryan, 2007, Lucki et al., 2001, David et al., 2003). BALB/c and C57BL/6 strains have both demonstrated

relatively high levels of baseline immobility in the FST compared to other strains (Lucki et al., 2001, Jacobson and Cryan, 2007). Furthermore, both BALB/c and C57BL/6 mice have demonstrated an antidepressant response (reduction in immobility) in response to various antidepressants, including fluoxetine, paroxetine and desipramine (Lucki et al., 2001, David et al., 2003).

There have been different hypotheses for the meaning of immobility in the FST, from a reduction in escape-directed behaviour (behavioural despair) to an alteration in coping strategies in stressful situations (Cryan et al., 2002, Cryan and Mombereau, 2004). The FST shows one of the highest levels of predictive validity of all animal models of depression, and is reliable and reproducible between and within laboratories, making it one of the most widely used tests of depression-related behaviour (Cryan et al., 2002). A reduction in immobility in the FST has been observed following both acute administration of antidepressants (Cryan et al., 2002), as well as chronic antidepressant treatment (Cryan et al., 2005b, Dulawa et al., 2004).

However, there are some limitations of the FST. While it shows predictive validity, face and construct validity are minimal. Furthermore, it has been suggested that considering immobility seen in the FST as behavioural despair may be anthropomorphic, and that as immobility is best for energy conservation it may reflect a more efficient way of coping (Powell et al., 2012).

There has been some debate as to whether the FST would be better described as a test of antidepressant action, rather than depression-related behaviour, given that it was developed and validated based on the effects seen with antidepressant drugs available at the time (Cryan and Mombereau, 2004). However, it has been shown that the FST not only demonstrates the effects of antidepressants, but also shows a pro-depressive effect (increased immobility) in rodents following interventions known to cause depression in humans, such as genetic predisposition, stress and withdrawal from psychostimulants

(Cryan and Mombereau, 2004). This can be seen to further validate its role as a test of depression-related behaviour.

5.1.2 Sucrose Preference Test

The sucrose preference test (SPT) was first used by Willner et al. (1987), in rats undergoing chronic unpredictable mild stress, but has subsequently been used in mice following chronic stress (Strekalova et al., 2004). The SPT is a measure of anhedonia, one of the key symptoms of depression. The task involves giving animals a choice of drinking from two bottles, one containing water and the other containing a sucrose solution. Rodents have an innate preference for sucrose over water, although those displaying depressive-related behaviours following a periods of chronic stress show significantly reduced sucrose preference (Willner et al., 1987).

Stress-induced reductions in sucrose preference have been reversed by chronic treatment of several different antidepressant drugs, including fluoxetine, imipramine and desipramine, in both rats and mice (Willner et al., 1987, Bessa et al., 2013, Zhang et al., 2014), making the SPT a pharmacologically validated model of depression-related behaviour. However, there are limitations of the SPT. Strain differences of intake of low concentrations of sucrose have been reported, which correlate to differences in expression of a taste receptor gene (Lewis et al., 2005), suggesting innate differences in the ability to taste sucrose (Powell et al., 2012). As mice experience sucrose for the first time during the SPT, neophobia may confound the interpretation of changes in sucrose preference as anhedonia.

5.1.3 Elevated plus maze

The elevated plus-maze (EPM) is a test of anxiety-related behaviour, developed for use in the mouse by Lister (1987). It consists of two enclosed arms with high walls, and two open arms, arranged in the shape of a 'plus' around a central square. The entire maze is elevated above the floor. Mouse behaviour in the EPM is based on a conflict between exploration of a novel environment, and avoidance of the aversive open arms of the maze (Bourin et al., 2007). Mice placed in the centre of the maze will predominantly explore the closed arms, whilst avoiding the open arms. An anxiolytic effect is defined as an increase in both the proportion of time spent in the open arms, and the number of entries into the open arms (Treit et al., 2010).

The EPM is a pharmacologically validated test of anxiety-related behaviour, showing predictive validity for anxiolytic compounds. Drugs used clinically to treat anxiety in humans, such as benzodiazepines, reliably show an anxiolytic effect in the EPM (Treit et al., 2010). Conversely, the effects of antidepressant drugs on mouse behaviour in the EPM is conflicting. A number of studies have reported either an anxiolytic effect, an anxiogenic effect, or no effect, after either chronic or acute administration (Cole and Rodgers, 1995, Silva and Brandao, 2000, Treit et al., 2010). However, there are limitations of the EPM. It has been suggested that the EPM should be considered more a test of benzodiazepine drug action rather than a test of anxiety-related behaviour. In addition, changes in overall activity levels may confound the results.

5.1.4 Behavioural effects of chronic stress

As chronic stress is well known to increase the risk of depression in humans, there has been considerable interest in assessing the behavioural effects of chronic stress in rodents. Various chronic stress paradigms have been developed, predominately using

adult animals, which have been shown to increase depression and anxiety-related behaviours in the FST, SPT and EPM. An increase in immobility in the FST, reflective of an increase in depression-related behaviour, has been shown following social stress (exposure to an unfamiliar male mouse), restraint stress and chronic mild stress (Huang et al., 2013, Kim and Han, 2006, Zhu et al., 2014). Similarly, in the EPM, social stress, restraint stress and chronic mild stress have all resulted in a decrease in the number of open arm entries, or time spent in the open arms, indicating an increase in anxiety-related behaviour (Kim and Han, 2006, Schmidt et al., 2007, Stone and Quartermain, 1997, Zhu et al., 2014). Chronic mild stress paradigms have frequently been shown to reduce preference for sucrose, indicative of anhedonia, in the SPT (Strekalova et al., 2004, Willner et al., 1987, Zhu et al., 2014).

While the behavioural effects of various chronic stress paradigms have been well validated in adult animals, there are relatively few reports of the behavioural effects of chronic stress in juvenile animals. In addition, where the effects of juvenile stress have been investigated, behaviour is often assessed when mice have reached adulthood (Huang et al., 2013, Schmidt et al., 2007, Brydges et al., 2014).

5.1.5 Chapter aims

This chapter aims to assess the behavioural effects of chronic stress in both juvenile and adult mice. The effects of 3, 7 and 14 days repeated restraint stress on depression and anxiety-related behaviours in the FST, SPT and EPM was determined in both juvenile and adult BALB/c mice. As the BALB/c mouse strain has been reported to be relatively stress sensitive compared with the more resilient C57BL/6 strain (Jacobson and Cryan, 2007), the behavioural effects of 3 days restraint stress in C57BL/6 adult and juvenile mice were also assessed.

In addition, as predictable stressors are considered less stressful than unpredictable stress (Anisman and Matheson, 2005), the changes in anxiety-related behaviour in the EPM following 3 days variable stress were determined in both BALB/c and C57BL/6 mice.

Finally, it was determined whether mice who exhibited a higher HPA response to stress, as shown by increased corticosterone release immediately following stress and increased adrenal gland weight, were more susceptible to the behavioural effects of stress. For each individual mouse, corticosterone levels and adrenal gland weight, as measured in Chapter 4, were correlated with behavioural readouts in the FST, SPT and EPM. These correlations were very exploratory analyses to look for patterns in the data, given that we had behavioural and neuroendocrinological measures from the same animals. However, the power is limited due to the small sample size.

5.2 Methods

5.2.1 Animals

To assess the behavioural effects of repeated stress in both juvenile and adult mice, BALB/c and C57BL/6 mice underwent either 3, 7 or 14 days restraint stress, or 3 days variable stress, as described in Chapter 2.3. The behavioural effects of acute stress, and of a shorter duration of restraint (30 min/day for 3 days) were also assessed. Behavioural testing in the EPM and FST occurred in a dimly lit room adjacent to the animal holding room, between 09:00-14:00h. Mice were left to acclimatise in the behavioural room for at least 1 hour prior to testing. Testing in the EPM, FST and SPT occurred one day following 3 days restraint to determine whether there was any lasting impact on the behaviour of the animal from repeated restraint stress. Behavioural testing occurred two days following 7 or 14 days restraint to allow for blood sampling 24h after restraint. Separate groups of mice were used for each behavioural test for ethical reasons and to avoid confounds due to prior behavioural testing. The use of the same animals for behavioural testing and neuroendocrinological analysis resulted in a reduction in the total number of animals used, as well as enabling comparisons of the behavioural and neuroendocrine measures for each individual mouse. An outline of the experimental protocols used are shown in Figure 5.1.

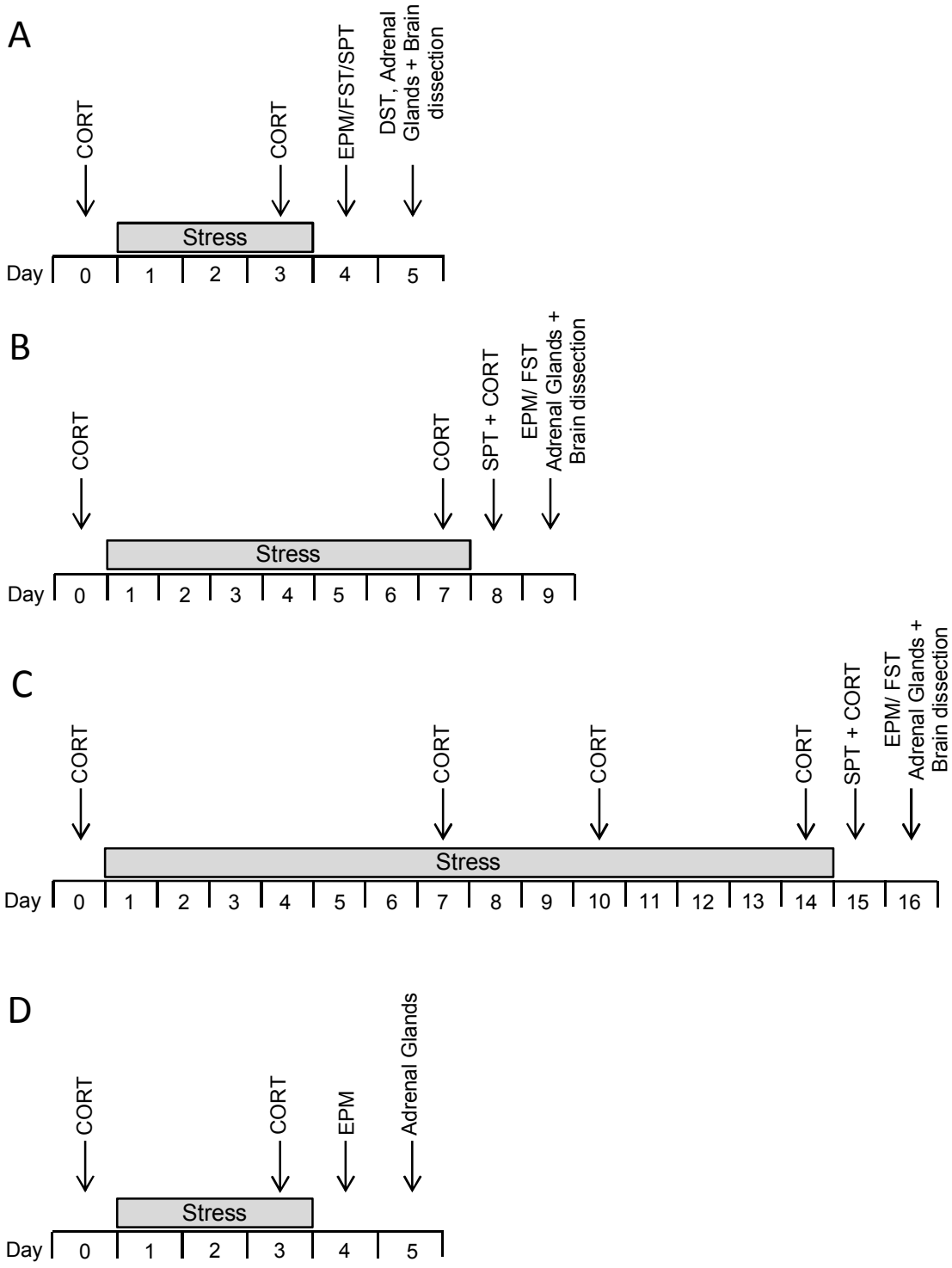

Figure 5.1: Experimental design for mice undergoing either 3, 7 or 14 days restraint stress (A, B and C), or 3 days variable stress (D). CORT, blood taken for corticosterone analysis; Brain dissection, brain tissue taken for molecular analysis; DST, dexamethasone suppression test; EPM, elevated plus maze; FST, forced swim test; SPT, sucrose preference test.

5.2.2 Forced swim test (FST)

The forced swim test (FST) was carried out essentially as described by Lucki (1997). Mice were placed in a glass beaker (diameter 22cm, height 34cm), filled to a depth of 23cm with water (25 ± 1°C). Each 6 minute test session was recorded using a camcorder (Sony DCR-SR52) and behaviour in the last 4 minutes of the test was scored by an experimenter blind to treatment. Swimming was defined as horizontal movement, climbing as vertical movement, while immobility was defined as the minimal activity needed to stay afloat (Cryan et al., 2002). Following the test session, mice were dried with paper towels and placed in a warm holding cage, before being returned to the home cage. The water was replaced, and the beaker cleaned with 70% ethanol, between each mouse.

5.2.3 Sucrose preference test (SPT)

The sucrose preference test was adapted from methods described by Willner et al. (1987). All mice undergoing the SPT were housed individually for at least 3 days prior to testing. Mice were first habituated to drinking from 2 bottles of water in the home cage, for 12h (19:00-07:00). The following day, mice were given the choice to drink either water or 2.5% or 5% w/v sucrose (D-sucrose, Fisher) in the home cage in a 12h test during the dark phase of the light cycle (19:00-07:00). Bottles were weighed before and after the test, and the preference for sucrose was determined as a percentage of the total volume of sucrose and water consumed.

5.2.4 Elevated plus maze (EPM)

The elevated plus maze (EPM) has been previously been used as a test to measure anxiety-related behaviour in the mouse (Lister, 1987). The EPM (CM EPM2000 Mouse Plus Maze, Campden Instruments) consisted of four arms (38 x 5cm), arranged at right angles around a central intersection (5 x 5cm). Two of the arms were open, with a 0.5cm rim, whilst the other two were enclosed with 9.5cm high walls. The entire maze was elevated 65cm off the floor. Lighting on the open arms measured 20 lux for experiments with BALB/c mice, and 50 lux for experiments using C57BL/6 mice, with light levels of less than 1 lux in the closed arms. The lighting conditions were determined empirically and the aversive nature of the open arms was validated pharmacologically using 1mg/kg diazepam as a positive control. Mice were placed in the central intersection facing an open arm and allowed to freely explore the maze for 5 minutes. Time spent in, and number of entries into the open arms, and total locomotion over the whole maze, were recorded by MotorMonitor™ software using infrared photobeams. The maze was cleaned with 70% ethanol after each mouse.

5.2.5 Statistical analysis

All statistical analysis was performed using InVivoStat software Version 2.5.0.0 (Clark et al., 2012). Elevated plus maze and forced swim test experiments were conducted as separate experiments in adult and juvenile mice, so data were analysed using unpaired t-tests to compare the effects of stress with non-stressed control mice. Sucrose preference tests in adult and juvenile mice were conducted as one experiment, and so were analysed using two-way ANOVA with age and stress as factors. Least significant difference (LSD) test with Bonferonni's correction for multiple comparisons was used for post-hoc comparisons. All sample sizes are indicated in the figure legends. All data are presented as mean ± SEM, and significance was taken as $P<0.05$.

5.3 Results

5.3.1 Validation of behavioural tests in juvenile mice

Initial experiments aimed to validate the suitability of the FST, SPT and EPM for use with both adult and juvenile BALB/c mice. During preliminary studies the effect of chronic fluoxetine treatment (20mg/kg i.p. for up to 28 days) was investigated. An adverse effect was noted on the seminal vesicles following treatment (see appendix).

5.3.1.1 Validation of the FST

The effect of acute fluoxetine (10 and 20mg/kg) on behaviour was assessed in adult and juvenile BALB/c mice (Figure 5.2). Fluoxetine treatment had a significant effect on both the time spent immobile and swimming ($F_{(2,40)}$=4.1, P=0.02). In juvenile mice, 10 and 20mg/kg fluoxetine resulted in a 24% decrease in the time spent immobile, and a 30% increase in the time spent swimming compared with saline-treated mice, which had a trend towards statistical significance (P=0.1). In adult mice, fluoxetine resulted in a 10% decrease in the time spent immobile, and 25% increase in the time spent swimming, although this was not statistically significant. There was also a significant effect of age on both the time spent immobile and swimming in the FST ($F_{(1,40)}$=26, P<0.001), with juvenile saline-treated mice spending 20% less time immobile, and 45% more time swimming, than adult saline-treated mice. Time spent climbing was found to be negligible, with an average of less than 0.5s in all groups.

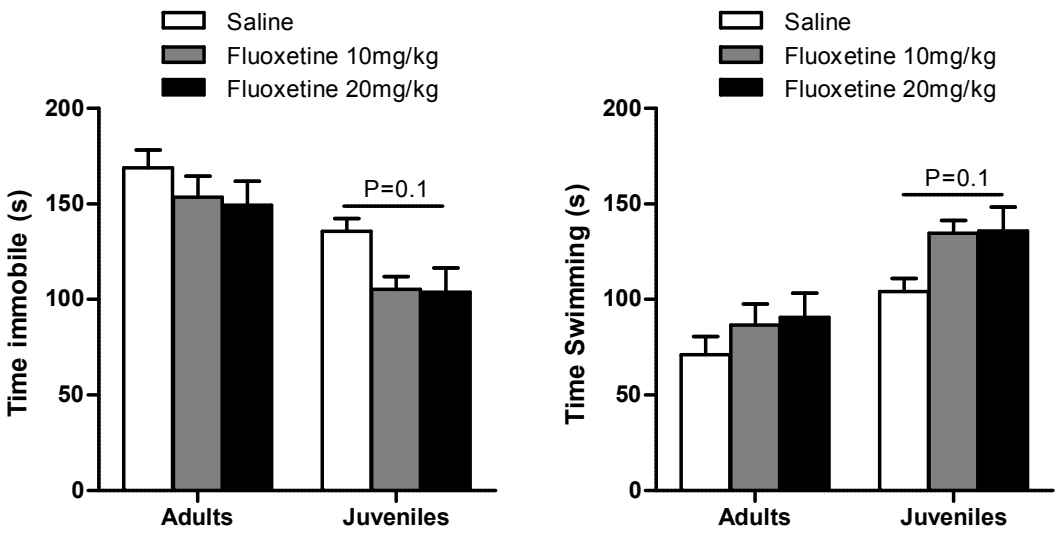

Figure 5.2: Effect of fluoxetine on behaviour of adult and juvenile BALB/c mice in the FST. Time spent immobile and swimming were recorded in the last 4 minutes of a 6 minute test. Test session began 30 minutes after i.p. administration (10ml/kg) of either 10 or 20 mg/kg fluoxetine, or 0.9% w/v saline. Results are expressed as mean ± SEM, n=8/group (post-hoc LSD test).

5.3.1.2 Validation of the SPT

In preliminary experiments, the innate preference of BALB/c mice to drink from a bottle positioned at either the front or the back of the cage was determined. Mice were given the choice to drink from two bottles, both containing water, in a 12h test period during the dark phase of the light cycle (19:00-07:00), and their preference for each bottle position was determined (Figure 5.3A). Mice had no significant preference for either the front or back bottle ($F_{(1,22)}$=0.5, P=0.5). Average total consumption was 3.4 ± 0.2ml for adult mice, and 3.7 ± 0.1ml for juvenile mice (mean ± SEM, n=12).

The ability of a 5% sucrose solution to produce a sucrose preference in BALB/c mice was also confirmed (Figure 5.3B). Mice were given the choice to drink from two bottles, one containing water and the other containing either water, or 2.5% or 5% w/v sucrose. A 2-way ANOVA revealed a significant effect of sucrose concentration ($F_{(2,18)}$=7.86, P=0.004) on preference for sucrose. Juvenile mice showed significant preference for 5% sucrose over water (P<0.05), with adult mice showing a trend towards a preference for 5% sucrose (P=0.1). 2.5% sucrose did not result in a significant preference over water in either adult or juvenile mice (Figure 5.3B).

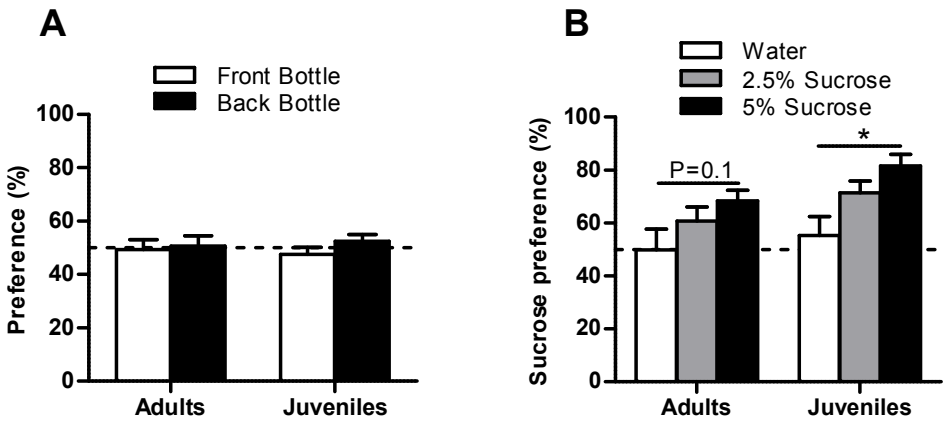

Figure 5.3: (A) Preference to drink from a water bottle positioned at either the front or back of the cage. Dotted line represents position of no preference. Results are expressed as mean ± SEM, n=12/group. (B) Preference for 2.5% or 5% sucrose solution, compared with water, in adult and juvenile BALB/c mice. Preference was measured over a 12h test period (19:00-07:00). Dotted line represents position of no preference. Results are expressed as mean ± SEM, n=4/group. *P<0.05 (post-hoc LSD test).

In C57BL/6 mice, previous work in our lab has established that consumption of 2.5% sucrose is significantly higher than water (P<0.01), and is sufficient to elicit a preference for sucrose in this strain of mouse (Figure 5.4, Wickens and Bailey, unpublished data).

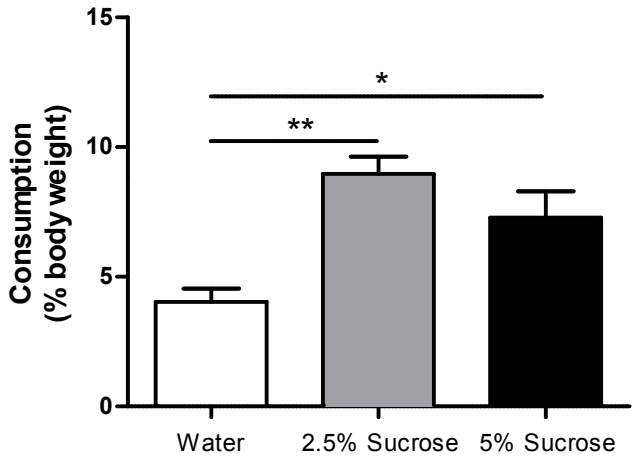

Figure 5.4: Consumption of water, 2.5% or 5% sucrose solution in adult C57BL/6 mice during a 1h test period at the beginning of the dark cycle (19:00h). Mice were water restricted for 4h prior to the start of the test. Results are expressed as mean ± SEM, n=4/group. *P<0.05, **P<0.01 compared with water (Dunnett's post-hoc test). Data from Wickens and Bailey (unpublished data).

5.3.1.3 Validation of the EPM

The anxiolytic effects of diazepam (1mg/kg) were evaluated in adult and juvenile BALB/c mice, under lighting conditions of 20 lux on the open arms (Figure 5.5). Diazepam had a significant effect on the time spent in the open arms ($F_{(1,27)}$=10.9, P=0.003), and resulted in a significant 300% increase in the time spent in the open arms in adult mice (P<0.001). There was also a significant effect of diazepam on the number of entries into the open arms ($F_{(1,27)}$=7.9, P=0.009), with a 175% increase in open arm entries in adult mice following diazepam treatment (P<0.01). Conversely, in juvenile mice diazepam had no significant effect on the time spent in, or entries into the open arms. Diazepam treatment resulted in a significant 130% increase in total locomotion over the whole maze in both adult and juvenile mice ($F_{(1,27)}$=61.4, P<0.0001).

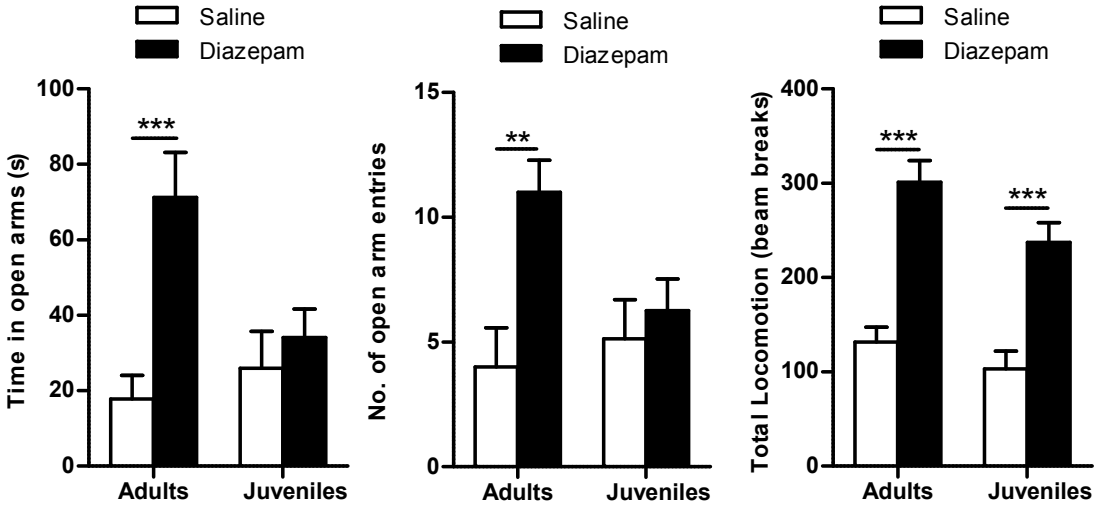

Figure 5.5: Effect of diazepam on behaviour of adult and juvenile BALB/c mice in the EPM. Time spent in the open arms, number of entries into the open arms and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test period. Test session began 30 min after i.p. administration (10ml/kg) of diazepam (1mg/kg), or 0.9% w/v saline. Results are expressed as mean ± SEM, n=8/group. **P<0.01, ***P<0.001 (post-hoc LSD test).

5.3.2 Effect of restraint stress on depression and anxiety-related behaviours

5.3.2.1 Forced Swim Test

Adult and juvenile BALB/c mice experienced daily restraint stress (2 hours per day) for either 3, 7 or 14 days. Behaviour in the FST was assessed 24-48 hours following the last restraint (Figure 5.6). A significant effect of restraint stress was only observed in adult mice following 3 days of restraint stress. This resulted in a 17% decrease in the time spent immobile, and a 35% increase in the time spent swimming in the FST ($P<0.05$, *t*-test). Extending the restraint stress for 7 or 14 days in adult mice had no significant effect on either the time spent swimming or immobile (all P's >0.3). In juvenile mice, behaviours in the FST were not affected by 3 or 7 days of restraint stress (all P's >0.6). Conversely, increasing the duration of restraint to 14 days resulted in a significant 27% decrease in the time spent immobile, and 37% increase in the time spent swimming in the FST ($P<0.05$, *t*-test).

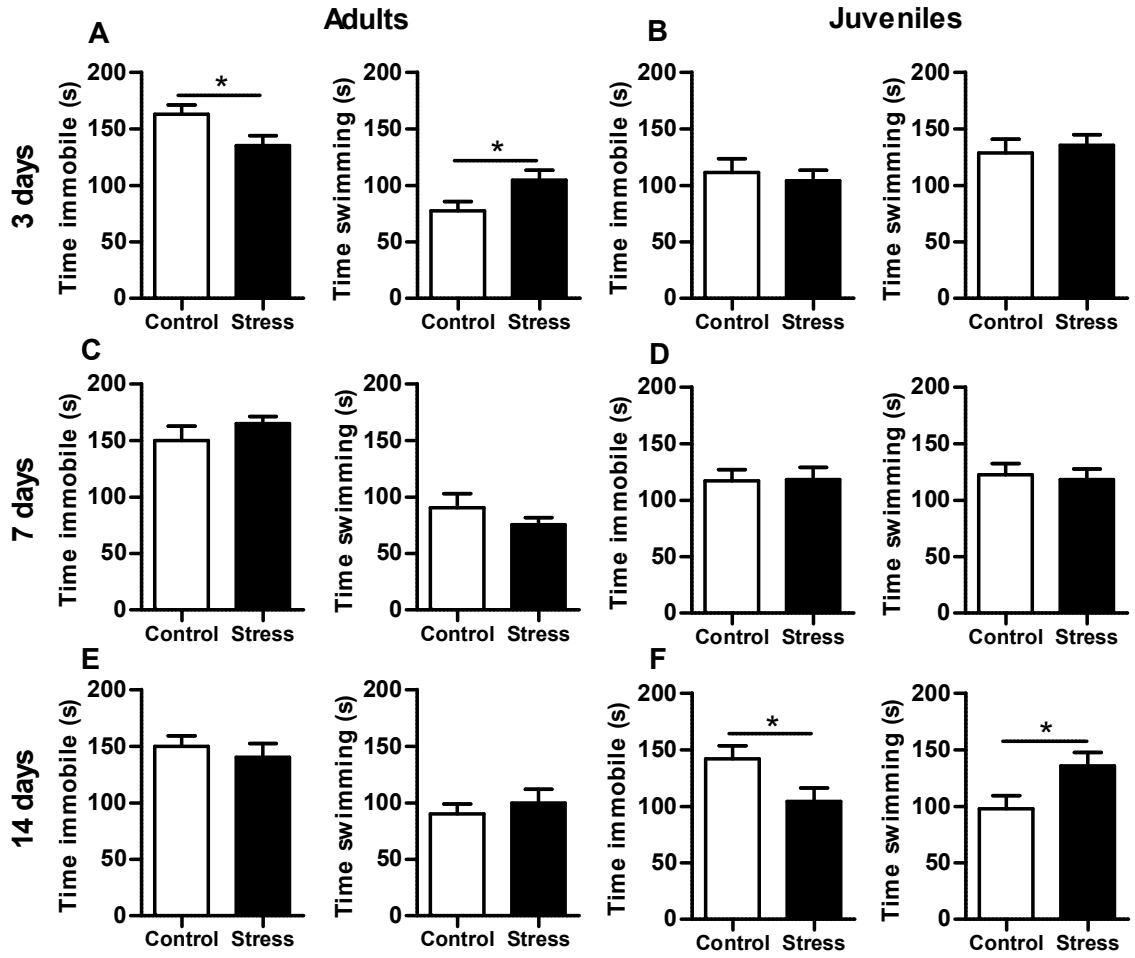

Figure 5.6: Effect of 3, 7 or 14 days restraint stress on behaviour in the FST in adult and juvenile BALB/c mice. Time spent swimming and immobile were recorded in the last 4 minutes of a 6 minute test. Results are expressed as mean ± SEM, n=7-12/group. *P<0.05 compared to control (unpaired *t*-tests).

5.3.2.2 Sucrose Preference Test

The effect of 3, 7 and 14 days restraint stress in BALB/c mice was also assessed in the SPT (Figure 5.7). The effect of both stress and age on sucrose preference was determined for each duration of stress using a two-way ANOVA, with age and stress as factors. There was a significant main effect of stress in 3 days restraint stressed mice ($F_{(1,25)}$=4.58, P<0.05). While this was associated with a 10% decrease in sucrose preference in both adult and juvenile stressed mice compared with control, this was not statistically significant following pairwise comparisons (P=0.2). There was no significant difference in total consumption of both water and sucrose between stressed and control mice in either adults or juveniles following 3 days restraint (P's>0.1). Following 7 days stress, there was a significant effect of stress ($F_{(1,27)}$=7.29, P<0.05), age ($F_{(1,27)}$=7.91, P<0.01) and age*stress interaction ($F_{(1,27)}$=5.35, P<0.05) on sucrose preference. In adults, there was a 33% increase in sucrose preference in stressed mice compared with controls (P<0.01). There was no effect of 7 days stress on sucrose preference in juvenile mice. There was no effect of 7 days restraint on total consumption ($F_{(1,27)}$=0.75, P=0.4). 14 days restraint resulted in a 12% reduction in sucrose preference in adult mice, and a 16% reduction in juveniles ($F_{(1,26)}$=16.59, P<0.001). This was accompanied by a significant decrease in consumption in juvenile, but not adult, stressed mice compared with control (P<0.05).

Repeated restraint stress therefore provoked an apparent antidepressant-like effect on behaviours in the FST after 3 days restraint stress in adults or 14 days restraint stress in juveniles. However, in the SPT, the behavioural response to repeated stress manifested as a significant decrease in preference for sucrose in juveniles, and a trend towards a significant decrease in adults, suggestive of an anhedonic or pro-depressant response to 14 days restraint stress.

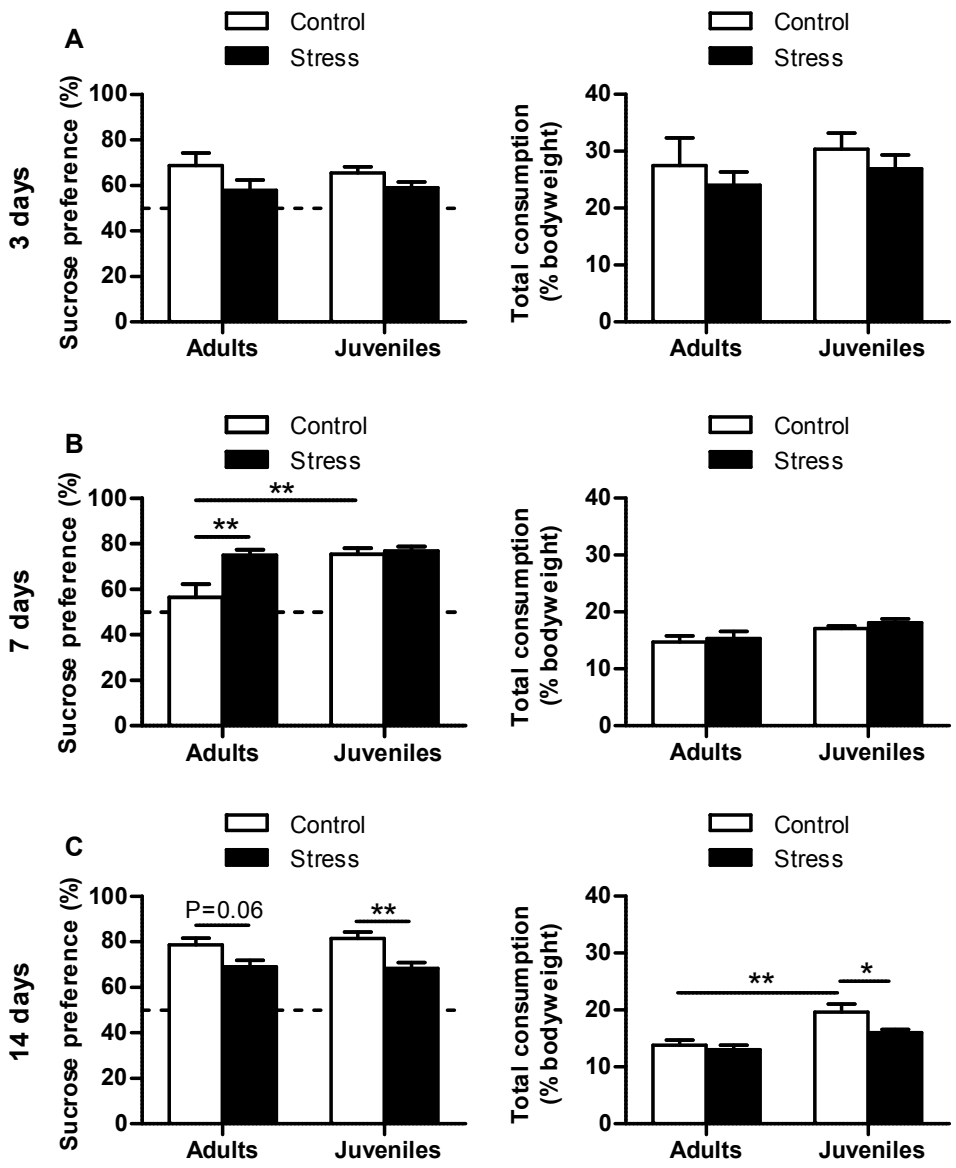

Figure 5.7: Effect of 3, 7 or 14 days restraint stress on sucrose preference in adult and juvenile BALB/c mice. Preference for 5% sucrose solution, and total consumption of both sucrose and water were measured over a 12h test period (7pm-7am). Dotted line represents position of no preference. Results are expressed as mean ± SEM, n=6-8/group. **P<0.01 (post-hoc LSD test).

5.3.2.3 Elevated Plus Maze

Separate groups of BALB/c mice underwent a similar restraint stress protocol and behaviour was evaluated in the EPM 24-48 hours following the last restraint session (Figure 5.8). In adult mice, only 7 days restraint stress resulted in a significant change in behaviour, evident as a 130% increase in the time spent in the open arms in stressed mice compared with control ($P<0.05$). However, in juvenile mice, 3 days restraint resulted in a 200% increase in the number of open arm entries ($P<0.01$), although this was associated with a 160% increase in locomotor activity ($P<0.001$). There was no change in the time spent in the open arms ($P=0.8$). 7 days restraint resulted in a 220% increase in the time in the open arms ($P<0.05$), and a 170% increase in the number of open arm entries ($P<0.01$). A significant 71% increase in locomotor activity was evident following 14 days restraint ($P<0.01$) in juvenile mice (but not in adults), although there was no change in either the time spent in, or entries into, the open arms of the EPM (both P's >0.1, Figure 5.8).

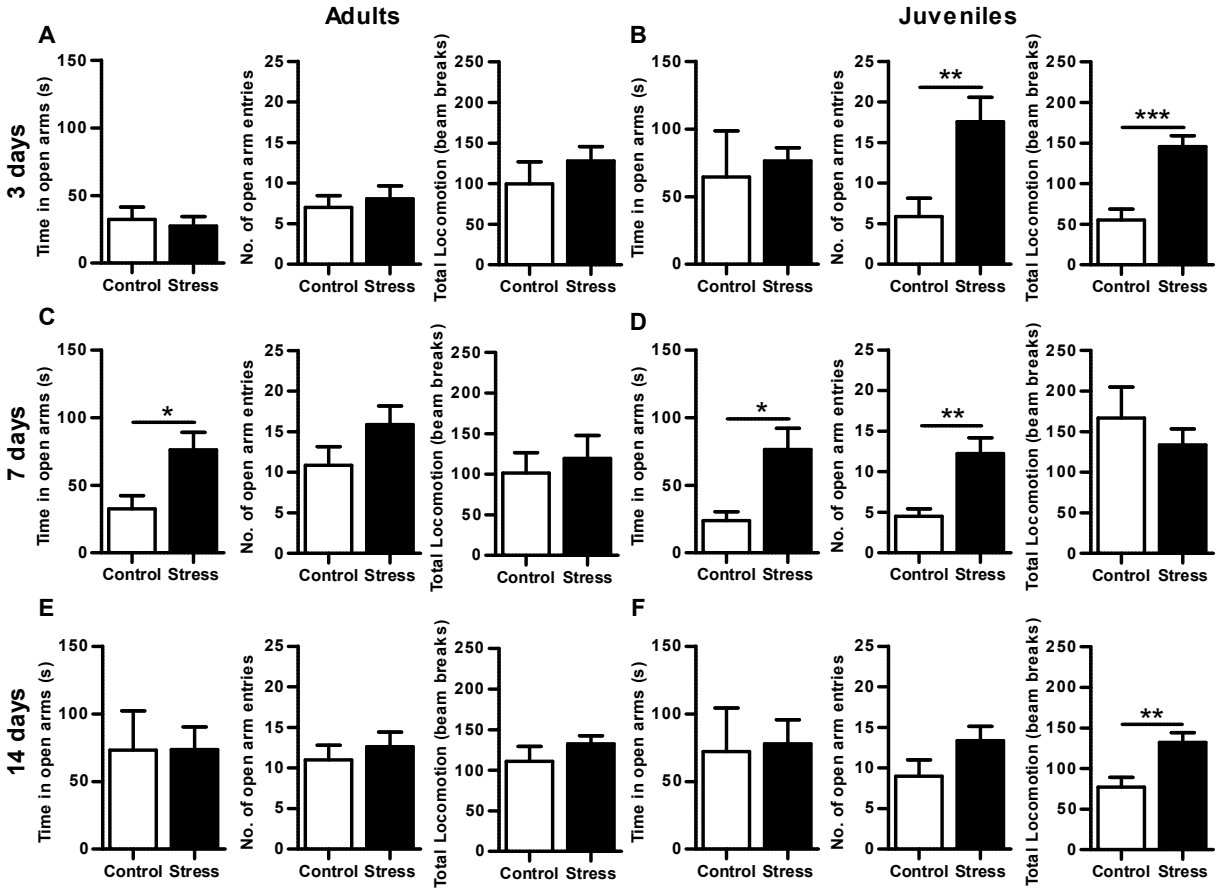

Figure 5.8: Effect of 3, 7 or 14 days restraint stress on behaviour in the EPM in adult and juvenile BALB/c mice. Time spent in the open arms, number of entries into the open arms and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test period. Results are expressed as mean ± SEM, n=7-12/group. *P<0.05, **P<0.01, ***P<0.001 (unpaired t-tests).

To determine whether a shorter duration of restraint led to less habituation to the stressor following repeated stress, the effect of 30min restraint for 3 days on behaviour in the EPM was assessed in BALB/c adult mice (Figure 5.9). There was no significant effect of 30min/day restraint stress on time spent in the open arms or the number of open arm entries, which was similar to results seen following 2h/day restraint stress for 3 days in adult mice. Conversely, there was an 80% increase in total locomotion following 30min/day restraint stress, which showed a trend towards statistical significance (P=0.08). This was not evident following 2h/day restraint.

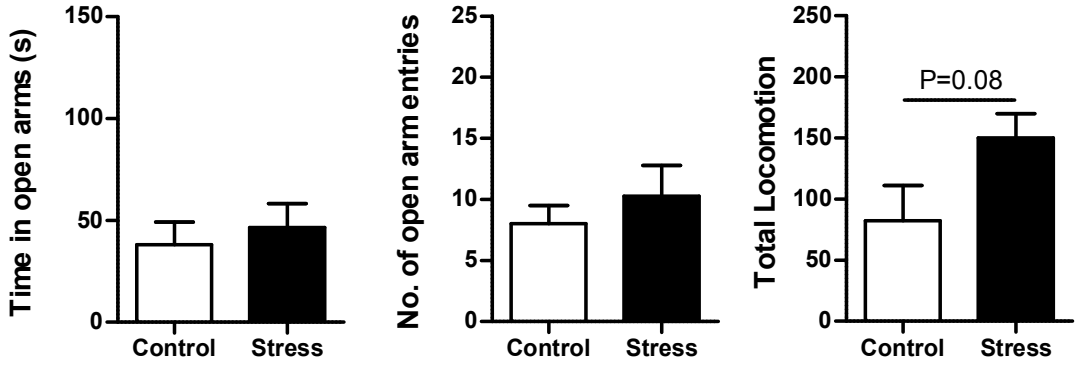

Figure 5.9: Effect of 3 days restraint (30min/day) on behaviour in the EPM in adult BALB/c mice. Time spent in the open arms, number of entries into the open arms and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test period. Results expressed as mean ± SEM, n=8/group (unpaired t-tests).

5.3.3 Behavioural effects of acute restraint stress

To compare the behavioural effects of repeated restraint stress with a single application of restraint, adult and juvenile mice were tested in either the FST, SPT or EPM the day after one 2h session of restraint (Figure 5.10). There was no significant effect of acute stress on the time spent either swimming or immobile in the FST (P=0.5), on sucrose preference (P=0.5), or on the time spent in the open arms, number of open arm entries, or total locomotion in the EPM (all P's >0.2).

In the FST, there was also a significant effect of age on the time spent both immobile and swimming ($F_{(1,,28)}$=19.3, P<0.001), with juvenile control mice spending 25% less time immobile than adults (P<0.01). There was no significant effect of age on sucrose preference ($F_{(1,25)}$=0.3, P=0.6), or on the time spent in the open arms ($F_{(1,28)}$=0.3, P=0.6) or number of open arm entries of the EPM ($F_{(1,28)}$=1.4, P=0.3).

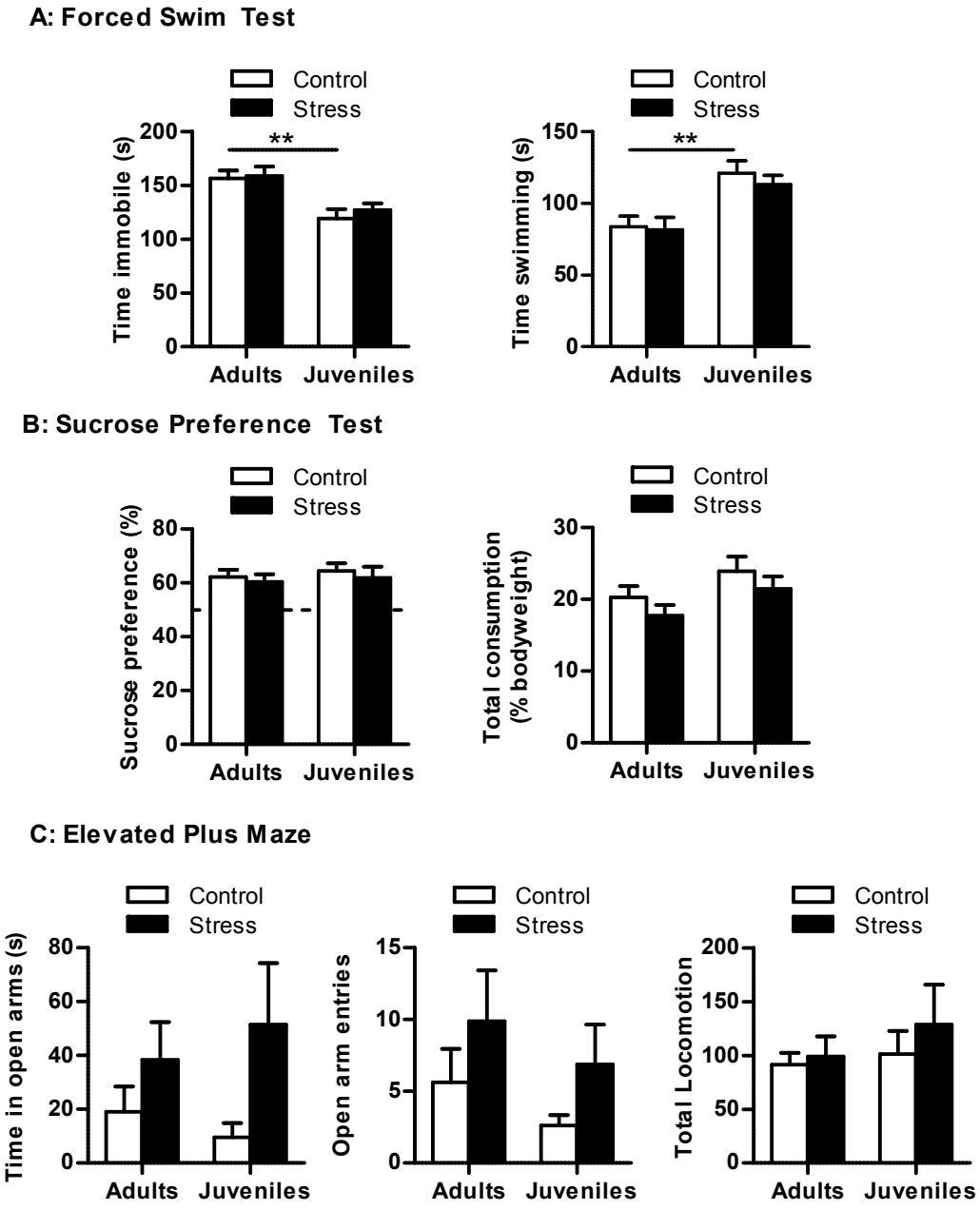

Figure 5.10: Effect of acute restraint stress (2h) on behaviour of adult and juvenile BALB/c mice in the forced swim test (A), sucrose preference test (B) and elevated plus maze (C). (A) Time spent immobile and swimming were measured in the last 4 minutes of a 6 minute test. (B) Preference for 5% sucrose solution, and total consumption of both sucrose and water were measured over a 12h test period (19:00-07:00). Dotted line represents position of no preference. (C) Time in the open arms, number of open arm entries and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test session. Results are expressed as mean ± SEM, n=7-8/group. **P<0.01 (post-hoc LSD test).

5.3.4 Effects of repeated restraint stress in C57BL/6 mice

The BALB/c mouse strain has been reported to be relatively stress sensitive whereas the C57BL/6 strain is relatively stress resilient (Jacobson and Cryan, 2007). Therefore the behavioural effects of 3 days restraint stress in C57BL/6 adult and juvenile mice were compared with BALB/c mice.

Following 3 days restraint stress, adult C57BL/6 mice displayed a 50% decrease in the time spent immobile, and a 65% increase in the time spent swimming in the FST ($P<0.01$, Figure 5.11). Conversely, in C57BL/6 juvenile mice, there was no effect of restraint on the time spent either immobile ($P=0.5$) or swimming ($P=0.6$).

In the SPT, a two-way ANOVA revealed a significant effect of age ($F_{(1,26)}=4.35$, $P<0.05$) on sucrose preference, but no significant effect of restraint stress ($F_{(1,26)}=2.79$, $P=0.1$) or stress*age interaction ($F_{(1,26)}=1.58$, $P=0.2$).

In the EPM, in juvenile mice restraint stress resulted in a 100% increase in the time spent in the open arms ($P<0.01$), consistent with an anxiolytic effect. Similarly, in adult mice, there was a 45% increase in time spent in the open arms following 3 days restraint, which had a trend towards statistical significance ($P=0.07$, Figure 5.11).

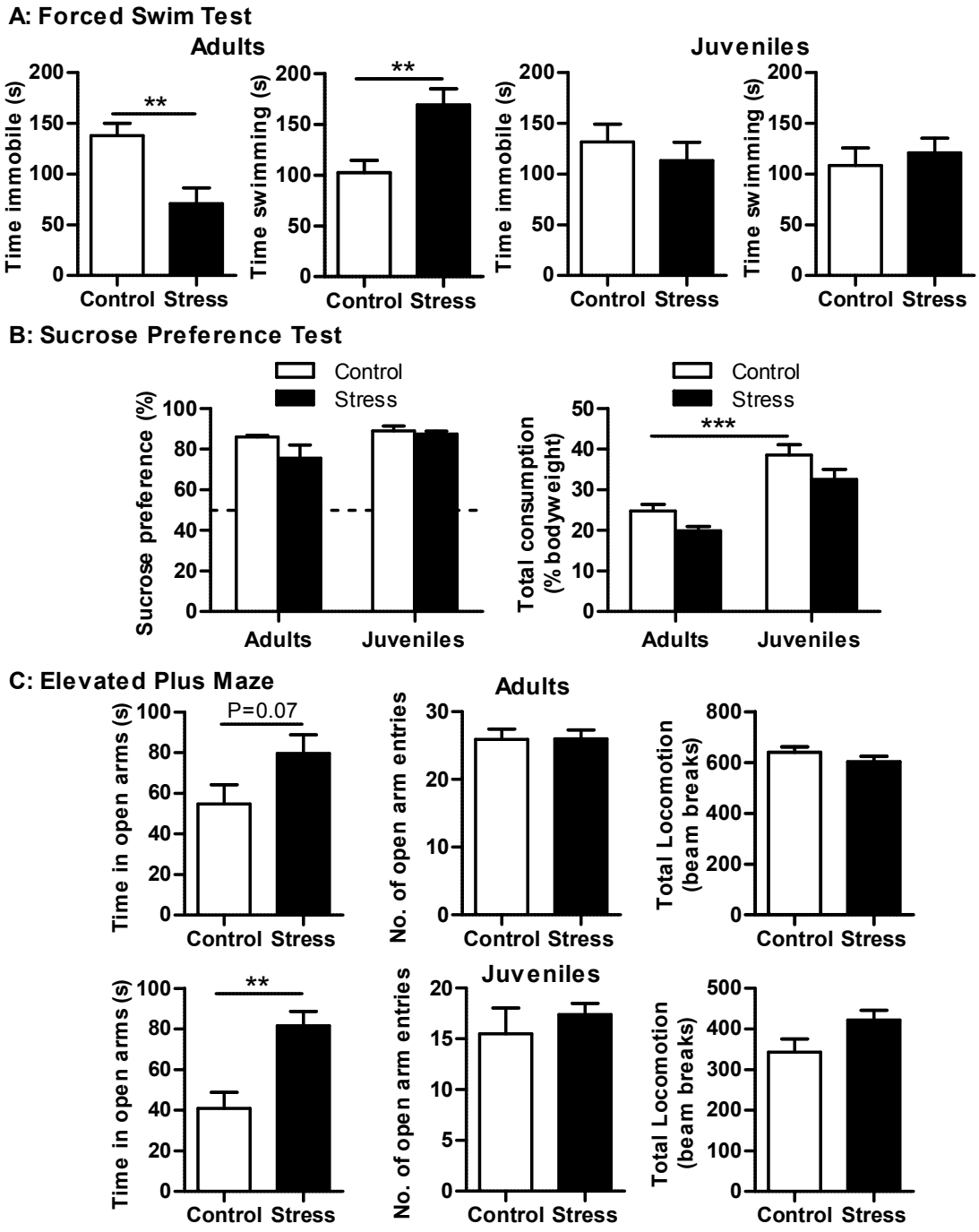

Figure 5.11: Effect of 3 days restraint on behaviour in the forced swim test (A), sucrose preference test (B) and elevated plus maze (C) in adult and juvenile C57BL/6 mice. (A) Time spent immobile and swimming were measured in the last 4 minutes of a 6 minute test. (B) Preference for 2.5% sucrose solution, and total consumption of both sucrose and water were measured over a 12h test period (19:00-07:00). Dotted line represents position of no preference. (C) Time in the open arms, number of open arm entries and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test session. Results are expressed as mean ± SEM, n=8-12/group. **P<0.01, unpaired t-tests (A,C), post-hoc LSD test (B).

5.3.5 Effect of juvenile stress on behaviour in adult mice

As the behavioural effects of chronic stress in juvenile mice have been shown to persist into adulthood (Tsoory et al., 2007), mice who had undergone 3 days repeated restraint stress when juvenile (4-5 weeks old) were retested in either the FST or EPM when they reached adulthood (9-10 weeks old). In the FST, there was no effect of juvenile stress on either the time spent immobile or swimming (P=0.6, Figure 5.12A). Similarly, in the EPM juvenile stress had no significant effect on either the time spent in, or entries into, the open arms (both P's>0.1, Figure 5.12B).

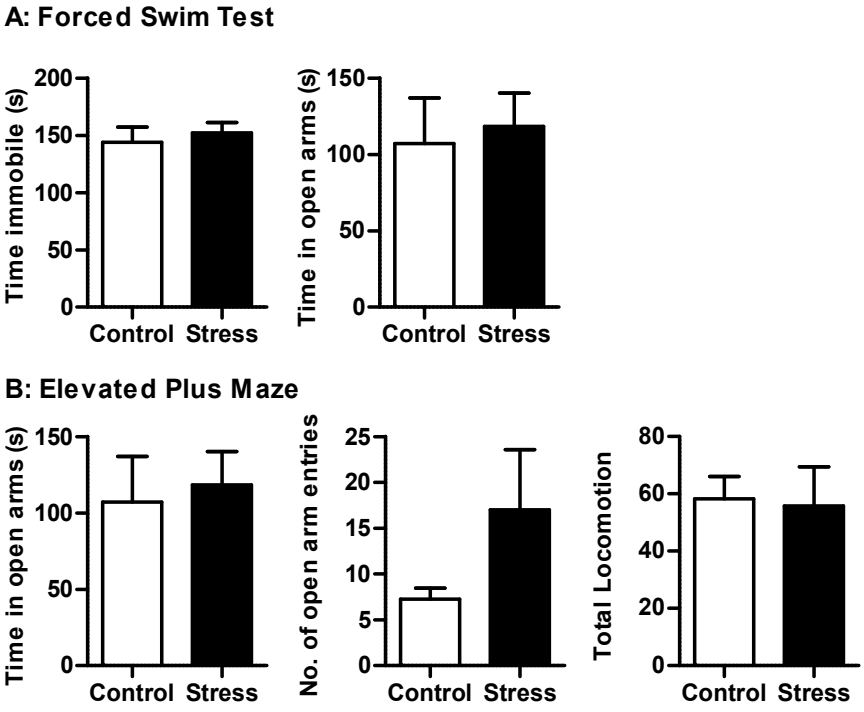

Figure 5.12: Effect of 3 days restraint stress in juvenile mice, on behaviour once mice reached adulthood. Testing in the forced swim test (A) and elevated plus maze (B) occurred 5 weeks after restraint stress. (A) Time spent immobile and swimming were measured in the last 4 minutes of a 6 minute test. (B) Time in the open arms, number of open arm entries and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test session. Results are expressed as mean ± SEM, n=7-8/group (unpaired t-tests).

5.3.6 Effect of variable stress on anxiety-related behaviours

As unpredictable stressors are considered somewhat more stressful than predictable stressors (Anisman and Matheson, 2005), the behavioural effects of 3 days variable stress were determined in the EPM (Figure 5.13). The effects of variable stress on behaviour in the FST were not examined due to the confounding effects of prior swim stress in stressed animals. Future studies would investigate the effects of variable stress on sucrose preference. The differences between the BALB/c and C57BL/6 strains were also directly compared.

In adult mice, there was a significant effect of strain on the time spent in the open arms ($F_{(1,28)}$=8.23, P=0.008), number of open arm entries ($F_{(1,27)}$=75.4, P<0.0001) and total locomotion ($F_{(1,29)}$=231.5, P<0.0001). C57BL/6 mice were significantly more active than BALB/c mice, showing almost 1000% higher overall locomotion and 650% more open arm entries (P<0.001). Similarly, in juvenile mice, C57BL/6 mice exhibited 600% higher overall locomotion ($F_{(1,30)}$=193.1, P<0.0001) and 600% more entries into the open arms ($F_{(1,30)}$=36.6, P<0.0001) than BALB/c mice (Figure 5.13).

In adult mice, variable stress had no effect on the time spent in the open arms ($F_{(1,28)}$=1.1, P=0.3) or open arm entries ($F_{(1,27)}$=3.2, P=0.09), although stress resulted in a significant 75% increase in total locomotion in BALB/c mice ($F_{(1,29)}$=5.7, P=0.02). Similarly, in juvenile mice, stress had no effect on time spent in the open arms ($F_{(1,30)}$=2.1, P=0.16), although it did result in a significant 400% increase in open arm entries ($F_{(1,30)}$=11.3, P=0.002) and 200% increase in total locomotion ($F_{(1,30)}$=34.6, P<0.0001) in BALB/c mice (Figure 5.13).

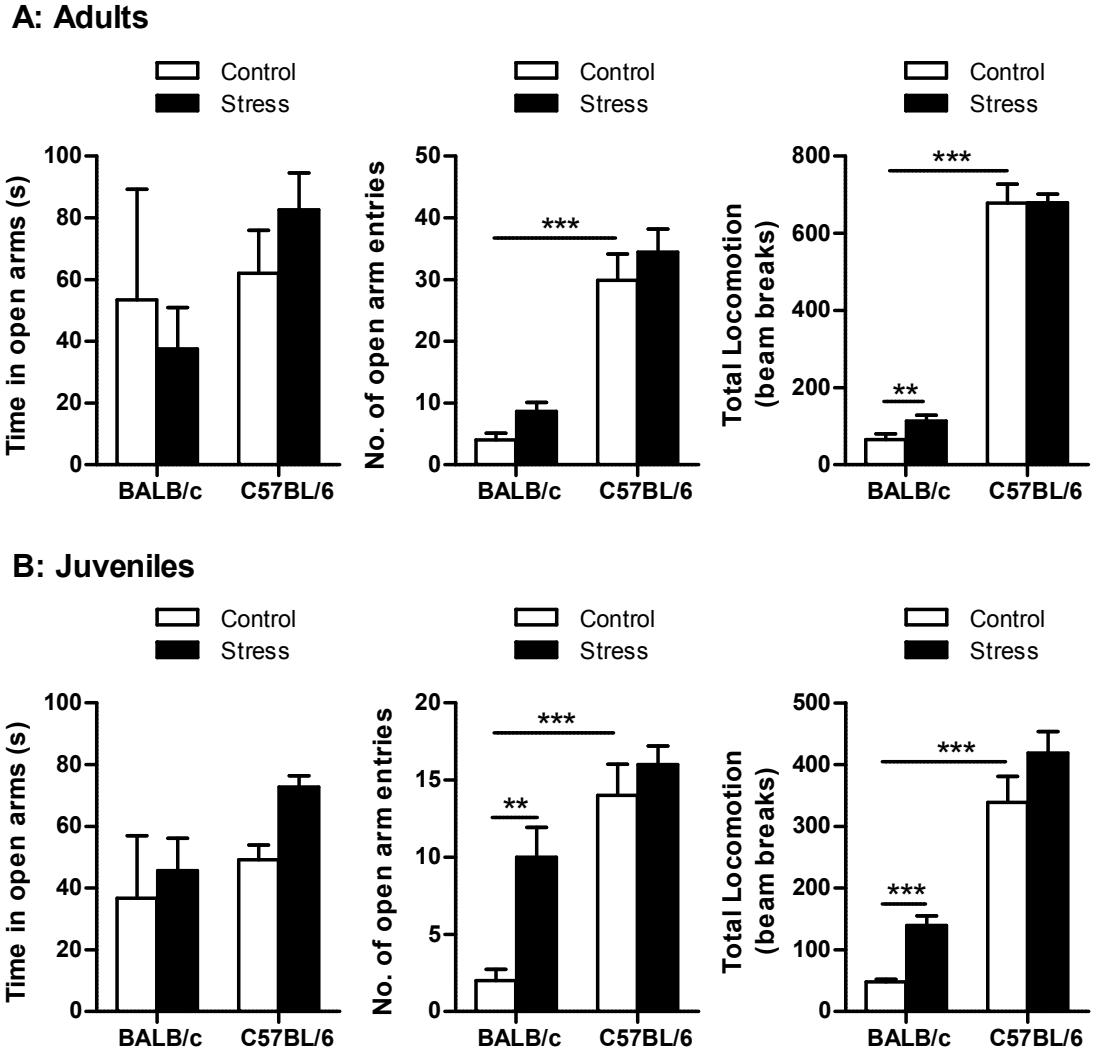

Figure 5.13: Effect of 3 days variable stress on behaviour in the EPM in adult (A) and juvenile (B) BALB/c and C57BL/6 mice. Testing in the EPM occurred the day after the last session of stress. Time in the open arms, number of open arm entries and total locomotion over the whole maze were measured in a 5 min test period. Results expressed as mean ± SEM, n=8-9/group. **P<0.01, ***P<0.001 (post-hoc LSD test).

5.3.7 Correlation of behavioural outcomes with measures of HPA activation

To assess whether mice who show increased HPA activity in response to stress are particularly susceptible to stress-induced changes in behaviour, behavioural outcomes in the FST, SPT and EPM were correlated with markers of HPA activation following stress. Pearson correlation coefficients were calculated between the HPA response (corticosterone levels immediately following stress, or adrenal gland weight, as described in chapter 4), and behavioural measures (either immobility in the FST, time spent in the open arms of the EPM, or sucrose preference) for each individual mouse. These were exploratory analyses to look for patterns in the data, and the power was limited due to the small sample size.

In the FST, there was a significant negative correlation between time spent immobile and post-stress corticosterone levels following 3 days restraint in BALB/c adult mice ($r= -0.43$, $n=24$, $P=0.04$) and C57BL/6 adult mice ($r= -0.56$, $n=24$, $P=0.005$). Conversely, a trend towards a significant positive correlation between immobility time in the FST and post-stress corticosterone levels was seen in BALB/c adult mice following 7 days restraint ($r=0.53$, $n=14$, $P=0.05$). No significant correlations were seen in BALB/c adult mice following 14 days restraint, or in juvenile mice at any timepoint following stress ($-0.3<r<0.2$, $n=15\text{-}16$, $P>0.2$, Table 5.1).

In the EPM, time spent in the open arms significantly correlated with post-stress corticosterone levels in adult BALB/c mice following 7 days restraint ($r=0.59$, $n=16$, $P=0.02$) and in C57BL/6 juvenile mice after 3 days restraint ($r=0.62$, $n=16$, $P=0.01$). There was a trend towards a positive correlation in C57BL/6 adult mice following 3 days restraint ($r=0.40$, $n=22$, $P=0.07$). No significant correlations were seen at any other timepoint following stress ($-0.2<r<0.4$, $n=15\text{-}22$, $P>0.1$, Table 5.1).

The number of open arm entries in the EPM also correlated with post-stress corticosterone in juvenile BALB/c mice following 3 days restraint ($r=0.55$, $n=15$, $P=0.04$).

No significant correlations were seen at any other timepoint following stress ($0.05<r<0.4$, n=16-23, $P>0.1$, Table 5.1).

In the SPT, there was a trend towards a significant negative correlation between preference for sucrose and post-stress corticosterone in BALB/c adult mice following 14 days restraint ($r= -0.48$, n=16, $P=0.06$). There were no significant correlations seen at any other timepoint following stress ($-0.5<r<-0.01$, n=6-16, $P>0.1$, Table 5.1).

Representative graphs showing an example of a positive correlation, negative correlation and no correlation are shown in Figure 5.14.

	Time spent in the open arms	Number of open arm entries	FST Immobility	Sucrose preference
3 days (BALB/c adults)	0	0	Negative	0
3 days (BALB/c juveniles)	0	Positive	0	0
7 days (BALB/c adults)	Positive	0	Trend to positive	0
7 days (BALB/c juveniles)	0	0	0	0
14 days (BALB/c adults)	0	0	0	Trend to positive
14 days (BALB/c juveniles)	-	-	-	0
3 days (C57BL/6 adults)	Trend to positive	0	Negative	-
3 days (C57BL/6 juveniles)	Positive	0	0	-

Table 5.1: Pearson correlation of corticosterone levels immediately following stress, and behavioural outcomes in the EPM, FST and SPT, for adult and juvenile BALB/c and C57BL/6 mice undergoing 3, 7 or 14 days restraint stress. 0, no significant correlation; positive, significant positive correlation; negative, significant negative correlation; -, no data.

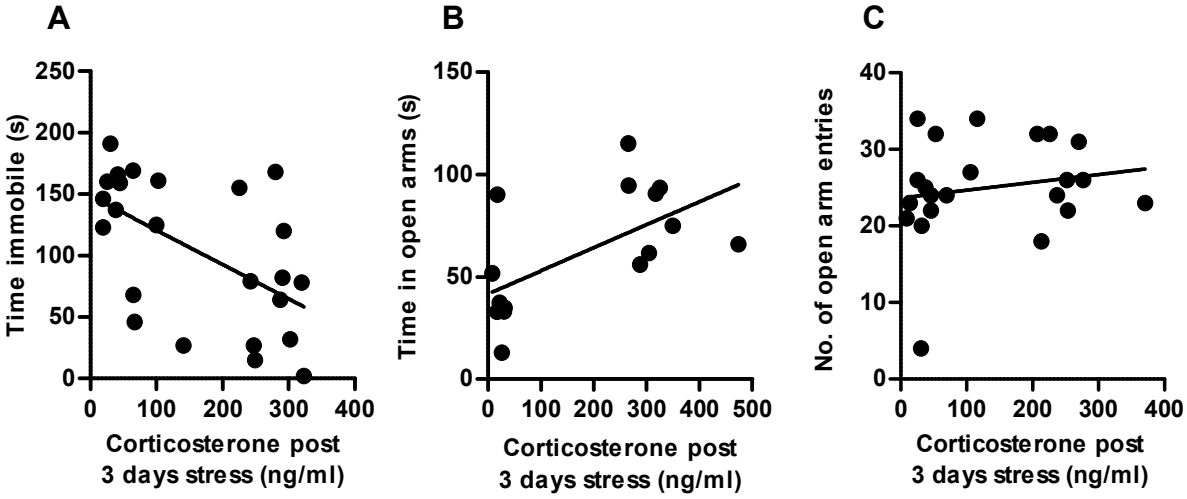

Figure 5.14: Representative graphs showing an example of a negative correlation, a positive correlation and no correlation. (A) Negative correlation between time spent immobile in the FST and post-stress corticosterone levels following 3 days restraint in C57BL/6 adult mice (r= -0.56, n=24, P=0.005). (B) Positive correlation between time spent in the open arms and post-stress corticosterone levels in C57BL/6 juvenile mice after 3 days restraint (r=0.62, n=16, P=0.01). (C) No correlation between the number of open arm entries and post-stress corticosterone levels in C57BL/6 adult mice after 3 days restraint (r=0.17, n=22, P=0.4).

Similar Pearson correlations were calculated between adrenal gland weight and behavioural outcomes (Table 5.2). In the FST, there was a significant negative correlation between the time spent immobile and adrenal gland weight in juvenile BALB/c mice following 14 days restraint (r= -0.57, n=14, P=0.04). A trend towards a negative correlation was seen in adult BALB/c mice following 3 days restraint (r= -0.71, n=7, P=0.07). No significant correlations were seen in juvenile mice, or at any other timepoint following stress ($-0.2<r<0.05$, n=15-23, $P>0.4$).

In the SPT, a significant negative correlation was seen between sucrose preference and adrenal gland weight in juvenile mice following 14 days restraint ($r= -0.69$, $n=16$, $P=0.003$). No significant correlations were seen at any other timepoints following stress ($-0.3<r<0.4$, $n=14-29$, $P>0.1$, Table 5.2).

In the EPM, there was a trend towards a significant correlation between adrenal gland weight and time spent in the open arms ($r=0.45$, $n=18$, $P=0.06$). There was also a trend towards a significant correlation between adrenal gland weight and number of open arm entries ($r=0.50$, $n=15$, $P=0.06$). No significant correlations were seen at any other timepoints following stress, between adrenal gland weight and either time spent in the open arms ($0.2<r<0.6$, $n=7-15$, $P>0.1$) or number of open arm entries ($-0.1<r<0.5$, $n=7-18$, $P>0.2$, Table 5.2).

Overall, while there are some behavioural outcomes in the FST, SPT and EPM which correlate with markers of HPA activation, there appears to be no consistent pattern as to which strain or age of mice, duration or type of stress, or which behavioural measure is significant. There seems to be a dissociation between neuroendocrine measures (which show a consistently increased HPA activation following stress), and behavioural measures (which do not show a consistent pattern).

	Time spent in the open arms	Number of open arm entries	FST Immobility	Sucrose preference
3 days (BALB/c adults)	0	0	Trend to negative	0
3 days (BALB/c juveniles)	-	-	-	0
7 days (BALB/c adults)	-	-	-	0
7 days (BALB/c juveniles)	-	-	0	0
14 days (BALB/c adults)	-	-	-	0
14 days (BALB/c juveniles)	0	Trend to positive	Negative	Negative
3 days (C57BL/6 adults)	Trend to positive	0	0	-
3 days (C57BL/6 juveniles)	0	0	0	-

Table 5.2: Correlation of adrenal gland weight following stress, and behavioural outcomes in the EPM, FST and SPT, for adult and juvenile BALB/c and C57BL/6 mice undergoing 3, 7 or 14 days restraint stress. 0, no significant correlation; positive, significant positive correlation; negative, significant negative correlation; -, no data.

5.4 Discussion

The behavioural changes seen in response to repeated restraint stress were complex. In the EPM, in juvenile mice, 3, 7 and 14 days of repeated restraint stress produced behavioural changes that included an *increase* in the number of open arm entries and/or time spent in the open arms, with and without changes in locomotor activity. This effect on locomotion in the EPM was only observed in adult mice following 7 days restraint, and no changes in the time spent in the open arms, or in the number of open arm entries were observed. Similar anxiolytic-like effects of repeated restraint stress were also evident in juvenile but not adult C57BL/6 mice after 3 days of exposure to the stressor. Interestingly, there was little effect of restraint stress on behaviours in the FST for BALB/c mice, where only the groups of adult mice with 3 days restraint stress, and juvenile mice with 14 days restraint stress, showed a *decrease* in time spent immobile, consistent with an antidepressant-like response. The same 3 day treatment in adult C57BL/6 mice also produced an antidepressant-like effect in the FST. In the SPT, a change in hedonic motivation was evident in both adult and juvenile BALB/c mice. Only after 14 days of repeated stress was a significant decrease in preference for sucrose observed, consistent with a pro-depressant phenotype.

One interpretation of these data is that the repeated restraint stress leads to a behavioural response that is an adaptive biological process. As a result of prior stress exposure, mice may adapt their behaviour as a stress-coping strategy. This behavioural change may be beneficial in future stress challenges. Thus, after repeated restraint stress, behaviour in the EPM manifests as an apparent anxiolytic-like response or behaviour in the FST manifests as an antidepressant-like response. Resilience in humans has been defined as the ability of an individual to avoid the negative effects of stress which may otherwise impact upon their mental or physical health (Russo et al., 2012). It has been shown that exposure to stress, particularly during childhood and adolescence, promotes the development of resilience (Lyons et al., 2010, Southwick and

Charney, 2012). In animals, resilience has been defined as the ability to avoid detrimental changes in behaviour following chronic stress (Russo et al., 2012). Resilience may be either an active, adaptive response, resulting in antidepressant and anxiolytic-like changes in behaviour (Russo et al., 2012, Suo et al., 2013), or a more passive mechanism, manifesting in an absence of pathological changes following chronic stress (Russo et al., 2012). The results shown here may reflect a mixture of both active and passive mechanisms of resilience, as shown by both an increased exploration of the open arms of the EPM following restraint stress in some cohorts of mice, as well as an absence of depression- or anxiety-related behaviours in others. The predictable nature of the restraint stress protocol used here may make it comparatively less stressful than unpredictable stressors (Anisman and Matheson, 2005), also contributing to the development of resilience rather than an anxious or depressive phenotype.

An alternative explanation for these data is that the increase in locomotor activity in the EPM may be an active response to stress, rather than a typical passive avoidance of the aversive open arms of the EPM. This could reflect an increase in a drive to escape the aversive environment (Mozhui et al., 2010). Increased locomotion in an open field has also been seen following restraint stress, and this stress-induced hyperlocomotion has been used as a marker of stress-responsivity in mice (Zimprich et al., 2014). An increase in mobility in the FST, and the similar tail suspension test, have also been reported following chronic stress, again suggesting an active defence mechanism in response to the aversive nature of the behavioural tests (Boulle et al., 2014). The increase in locomotion seen here in the EPM, and the reduction in immobility in the FST following stress, may be reflective of an active response to stress, rather than a reduction in depression and anxiety-related behaviours.

The differing nature of the stress-induced behavioural changes in the SPT, compared with the FST and EPM, may reflect the differing nature of each behavioural test. Both the FST and EPM assess a behavioural response to a stressful environment (a forced

swim or the open arms of the EPM), which may be useful for assessing stress-induced behavioural changes in response to aversive environments. Conversely, the SPT measures preference for a palatable substance (sucrose), in a non-stressful environment (the home cage). As such it may be a more suitable measure of depression-related behaviour following chronic stress. Importantly, in pilot studies pharmacological validation was conducted to assess the suitability of the FST and EPM for use in juvenile mice at the outset of this thesis. Acute administration of fluoxetine reduced the time spent immobile in the FST by juvenile but not adult mice (Figure 5.2). In the EPM, acute administration of diazepam increased the time spent in the open arms by adult mice but not juvenile animals, although total locomotion was increased in both age animals. A further complicating factor in interpreting these data is that the innate behaviour of juvenile mice in these tasks may be quite different from adult animals.

A particular strength of these studies is that the collection of multiple data from the same animal allowed the correlation of behavioural data in the FST, SPT and EPM with measures of HPA activation. While there was no consistent pattern of significant correlations, where significance was seen this generally indicated that increased HPA activation (higher post-stress corticosterone or increased adrenal gland weight) was associated with increased anhedonia in the SPT, and increased activity in both the FST and EPM. This indicates that increased HPA activation may make individual mice more susceptible to stress-induced changes in behaviour.

As unpredictable stress is considered more stressful than predictable stress (Anisman and Matheson, 2005), behavioural effects of a variable stress paradigm in the EPM were also examined. Similar to the data following repeated restraint stress, variable stress did not induce an anxious phenotype in the EPM, with no difference in the time spent in the open arms between stressed mice and controls. However, increases in locomotion were apparent in BALB/c mice following variable stress, and again this may be reflective of an active response to stress (Mozhui et al., 2010). In future studies it would be interesting

to investigate behaviour in the FST and SPT following a variable stress paradigm, to determine whether stress-induced behavioural changes can be observed in these assays. In this study we have compared the effects of restraint stress on two different strains of mice. There have been several studies looking at strain differences in stress responsiveness, with BALB/c mice considered to be more sensitive to the effects of stress than the more stress-resilient C57BL/6 strain (Anisman and Matheson, 2005, Jacobson and Cryan, 2007). Following 3 days restraint stress, hyperlocomotion was seen in the EPM in juvenile BALB/c mice, but not in C57BL/6 mice, reflecting increased sensitivity of BALB/c mice to stress. Similarly, increases in locomotor activity following 3 days variable stress were apparent in both adult and juvenile BALB/c, but not C57BL/6 mice, again reflecting the increased stress-sensitivity of the BALB/c strain. In the FST, 3 days restraint stress resulted in antidepressant-like reductions in immobility in both BALB/c and C57BL/6 adult mice, and this appeared more pronounced in C57BL/6 mice (50% decrease from control) than in the BALB/c strain (20% decrease from control). Again, this suggests increased resilience in C57BL/6 mice, compared to the more stress-sensitive BALB/c strain.

BALB/c mice used throughout these studies were housed individually to avoid fighting. The less aggressive C57BL/6 mice were housed in groups for EPM and FST studies, while C57BL/6 mice undergoing the SPT were housed individually. While the effect of housing conditions in the present study is unknown, it has previously been shown that individual housing of male BALB/c and C57BL/6 mice had no effect on either corticosterone levels or anxiety-related behaviour (Arndt et al., 2009), suggesting the effect of housing conditions may be limited. Another confound could be the frequency of cage cleaning, with cages of group housed mice cleaned weekly, whereas individually housed mice were cleaned fortnightly. However, as all mice were handled daily to reduce stress of handling, this may minimise any differences.

In conclusion, repeated restraint stress appears to induce a range of behavioural changes in both juvenile and adult mice. In neither age group tested was there a consistent behavioural phenotype that could be described as obviously “depression-like”. However, a range of behavioural responses were observed that may represent an adaptive behavioural response to repeated restraint stress.

6 Analysis of gene expression of HPA components in adult and juvenile mice

6.1 Introduction

The HPA axis plays a central role in the endocrine response to stress (Chapter 1.2). The HPA axis matures during adolescence and is important for normal stress reactivity (Romeo et al., 2016, Gunnar et al., 2009). For examples, early stages of neonatal life are characterized by a stress-hyporesponsive period in rodents. At this stage stress induces limited secretion of glucocorticoids (Levine, 1994, Schmidt et al., 2003). This early postnatal hyporesponsive period, within the first week of life, is thought to have a protective function in limiting the impact of high circulating glucocorticoids in the brain and is mediated in part by corticosteroid binding globulins and in part by GR mediated feedback (Schmidt et al., 2005). Pre-adolescent rats and mice (25-30 days old) show plasma ACTH and corticosterone response that lasted twice as long as those observed in adults (Romeo et al., 2016). The adult-like ACTH stress response develops during the later stages of adolescence (50-60 days) while the corticosterone response changes earlier at 30-40 days of age (Foilb et al., 2011). It is clear that during adolescence the HPA stress response develops to an adult pattern, however, the molecular changes underlying the maturation of the HPA stress response are not well understood.

The HPA stress response is also shaped by experience. Chronic stress has been reported to affect the functioning of the HPA axis. In humans, early life stressors, such as child abuse, can cause persistent changes and lead to HPA dysfunction (Heim et al., 2000, Heim et al., 2008, Tyrka et al., 2008). This is one mechanism which may account for the association of early life stress with the risk of developing major depressive disorder (Claes, 2009). In adult rats and mice exposed to repeated stress, the neuroendocrinological stress response habituates such that corticosterone responses are reduced (Grissom et al., 2007). In contrast, when juvenile animals are exposed to repeated restraint stress no habituation in their hormonal responses is observed (Lui et al., 2012, Romeo et al., 2016 and Chapter 4 of this thesis). However, this habituation to stress may be dependent on the nature of the stressor. Both adolescent and adult rats

have been shown to exhibit habituation to repeated episodes of social isolation (Hodges and McCormick, 2015). In this thesis, I have tested the hypothesis that changes in gene expression of components of the HPA axis underlie the differential response to stress observed in adult and juvenile animals. As this thesis principally investigates stress responses, gene expression analysis was targeted on components of HPA axis signalling rather than a genome wide microarray study.

This chapter aimed to determine whether expression of key components of HPA regulation are altered in juvenile mice compared with adults. Baseline differences in expression of AVP, CRH, CRHR1, CRHR2, GR, MR and V1b were determined in the hypothalamus, hippocampus, PFC and pituitary gland of juvenile and adult mice. These brain areas were selected as they are regions that they hypothalamus projects to, and are implicated in mood disorders (Pandya et al., 2012). It has been shown that expression of GR is similar in the hippocampus of prepubertal and adult rats, and that expression of CRH is greater in prepubertal rats than in adults (Romeo, 2010), although little is known about gene expression of components of the HPA axis in juvenile mice. Further experiments investigated the effect of repeated restraint stress on expression of components of the HPA axis changes in both juvenile and adult mice.

6.2 Methods

6.2.1 Dissection of brain regions

Following either restraint or variable stress and behavioural testing, mice were killed by cervical dislocation and decapitated. To avoid the acute effects of behavioural testing on gene expression, mice were killed 24h post-testing for gene expression analysis. Brains were removed, and microdissection of the PFC was performed by making a coronal cut (freehand with a razor blade, with the aid of a mouse brain atlas (Paxinos and Franklin, 2001) of the anterior portion of the brain. The entire hippocampus, hypothalamus and pituitary gland were also rapidly dissected. Brain tissues were placed immediately in tubes containing RNA*later*® (Applied Biosystems) and kept on ice. Samples were stored at 4°C overnight, then RNA*later*® was removed and samples were transferred to storage at -80°C until RNA extraction.

6.2.2 RNA isolation and quantification

RNA was extracted from brain tissue using TRIzol® (Invitrogen). 0.5ml TRIzol was added to the tissue sample which was homogenised with a pellet pestle (Sigma). A further 0.5ml TRIzol was added, and the homogenate passed through a 25G needle. 20µl glycogen (1mg/ml, Applied Biosystems) was added and the sample was left to stand at room temperature for 5 minutes. 200µl chloroform (Sigma) was added and samples were shaken vigorously by hand for 15s, left to stand for 2-3 minutes before centrifugation at 12000 rcf for 15 minutes at 4°C. The upper aqueous phase was removed and retained. RNA was precipitated by the addition of 0.5ml 100% propan-2-ol (Sigma) and incubation for 30 minutes at room temperature. Samples were centrifuged at 12000 rcf for 10 minutes, and the resulting pellets resuspended and washed in 1ml 75% ethanol (Sigma) before centrifugation at 7500 rcf for 5 minutes at 4°C. Pellets were air dried for 5-10

minutes, and re-suspended in 30µl RNase-free water (Applied Biosystems). Samples were then incubated at 55-60°C for 10 min.

A DNase digest was then performed to remove genomic DNA contamination. 4µl DNase I (1U/µl, Fermentas) 4µl 10X reaction buffer for DNase I (Fermentas), 1µl RiboLock RNase inhibitor (40U/µl, Fermentas) and 1µl RNase free water were added to RNA samples which and incubated for 30 min at 37°C. RNA was then reprecipitated by the addition of 10µl glycogen (1mg/ml) and 100µl 100% ethanol, and incubation for 10 min at room temperature. Samples were centrifuged at 12000 rcf for 15 min at 4°C, and the resulting pellets were washed with 250µl 75% ethanol. Samples were then centrifuged at 12000 rcf at 4°C, and the pellets then left to air dry at 37°C for 15 min. Pellets were resuspended in 20µl RNase-free water before quantification.

To determine the concentration of isolated RNA, samples were first diluted 1/25 into a total volume of 50µl RNase free water. Absorbance was then determined using a Biophotometer (Eppendorf) to measure absorbance at 260nm, where A_{260} =1 indicates a concentration of 40µg/ml. Purity of RNA samples was determined by measuring $A_{260/280}$ ratios, where $A_{260/280}$ of greater than 1.8 indicates a pure RNA sample.

6.2.3 One-step reverse transcription PCR

SuperScript® One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen) was used to determine the expression of genes of interest in specific regions of mouse brain (hippocampus, PFC, hypothalamus and pituitary gland), using the gene-specific primers outlined in Table 6.1. Primers were either derived from published sequences, from Primer-BLAST (Basic Local Alignment Search Tool, (Ye et al., 2012)) or from PrimerBank (Spandidos et al., 2010). 12.5µl 2X Reaction Mix (containing 0.4mM of each dNTP, and 2.4mM $MgSO_4$), 10.1µl RNase free water, 0.4µl RT/Taq mix, 0.5µl

forward primer (10μM), 0.5μl reverse primer (10μM) and 1μl template RNA (0.2μg/μl) were added to 0.2ml PCR tubes. A “no template” negative control was created by omitting the RNA from the reaction mixture, and replacing it with 1μl RNAse free water. The PCR tubes were placed in a PCR machine (MJ Research, PTC-200) under the following cycling conditions: 30 minutes at 50°C (cDNA synthesis), 2 minutes at 94°C (denaturation), 40 cycles of 15 seconds at 94°C (denaturation), 30 seconds at 60°C (annealing) and 30 seconds at 72°C (extension), followed by a final extension phase of 5 minutes at 72°C. The PCR products were then run on a 2% agarose gel containing 0.625μg/ml ethidium bromide (GeneChoice), and visualised using GeneSnap (SynGene) software.

6.2.4 Real-time quantitative reverse transcription PCR (qPCR)

6.2.4.1 Reverse Transcription

In order to quantify the expression of genes of interest in mouse brain regions, RNA was first reverse transcribed to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). 2μl 10x RT Buffer, 0.8μl dNTP mix (100mM), 1μl Reverse Transcriptase (50U/μl), 2μl Random Primers, 1μl RNase inhibitor (10U/μl, Fermentas), and 250ng RNA were added to a PCR tube and made up to a total volume of 20μl with RNase free water. Tubes were incubated at 25°C for 5 min, 37°C for 2h, and 85°C for 5 min in a PCR machine (MJ Research, PTC-200). The resulting cDNA (20μl containing 250ng original RNA input) was diluted in RNase free water to a final volume of 450μl, and stored at -20°C.

6.2.4.2 qPCR

For real-time quantitative reverse transcription PCR (qPCR), 9.2µl cDNA (containing 5.1ng original RNA input) was added to a 96 well PCR plate (MicroAmp®, Applied Biosystems) along with 10µl SYBR green PCR master mix (Applied Biosystems), and 0.4µl each of forward and reverse primers (10µM). Sequences of the primers used for qPCR are given in Table 6.1. A negative control was created by omitting the RNA from the reaction mixture. Aerosol barrier pipette tips were used to prevent cross-contamination. The plate was sealed with adhesive film (MicroAmp®, Applied Biosystems), and placed in a real-time PCR machine (Applied Biosystems StepOne Plus) under the following cycling conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and annealing temperature of 60°C for 1 minute, for all gene-specific primers. This was followed by melt curve analysis to check the specificity of the primers and amplified PCR product. qPCR was performed in a 96 well plate format. The primary objective of this study was to identify differences between control and stressed groups in target gene expression. For logistical reasons it was not possible to include all genes of interest in all brain regions for both control and stressed groups on a single 96 well plate, so gene expression in different brain areas were not directly compared.

6.2.4.3 Comparative quantification cycle method ($2^{-\Delta\Delta Cq}$)

Differences in gene expression were quantified using the comparative quantification cycle method ($2^{-\Delta\Delta Cq}$) (Schmittgen et al., 2000). The quantification cycle (C_q) of the gene of interest in each sample was first normalised to the C_q of the reference gene phosphoglycerate kinase 1 (PGK1) to obtain ΔC_q. ΔC_q values for the control group were averaged, and subtracted from individual ΔC_q values for the stressed group to obtain $\Delta\Delta C_q$. This was then transformed to the equation $2^{-\Delta\Delta Cq}$ to obtain the fold change from the control group.

6.2.4.4 Determining reference genes

As expression levels of each gene of interest is normalised to a reference gene, it is important that the expression of any reference gene is stable across different experimental conditions (Bustin et al., 2009). Studies have shown that the expression of phosphoglycerate kinase 1 (PGK1) is stable throughout development of the mouse brain, and hence may be suitable for use as a reference gene in studies comparing gene expression in mice of different ages (Boda et al., 2009). The stability of three reference genes, 18S ribosomal RNA (18S), β-actin, and PGK1 in adult and juvenile mice, across different brain regions, was established at the outset of the qPCR experiments (See Table 6.1 for primer sequences). Stability of expression was determined using both GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) softwares.

6.2.5 Statistical analysis

All statistical analysis was performed using InVivoStat software Version 2.5.0.0 (Clark et al., 2012). Comparisons of gene expression in adult and juvenile naïve mice were analysed using unpaired t-tests. The effect of stress on gene expression was analysed using a 2-way ANOVA to determine differences in stressed vs. control mice, as well as adult vs. juvenile mice, for each gene of interest at each duration of stress. Least significant difference (LSD) test with Bonferonni's correction for multiple comparisons was used for post-hoc comparisons. All sample sizes are indicated in the figure legends. All data are presented as mean ± SEM, and significance was taken as $P<0.05$.

Primer	Sequence	Amplicon Length (base pairs)	References
18S rRNA	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	152	Schmittgen and Zakrajsek (2000)
β-actin	F: ACCAACTGGGACGATATGGAGAAGA R: TACGACCAGAGGCATACAGGGACAA	214	Schmittgen and Zakrajsek (2000)
PGK1	F: ATGTCGCTTTCCAACAAGCTG R: GCTCCATTGTCCAAGCAGAAT	164	Primer Bank
CRH	F: GGCCCCGCAGCCCTTGAATTT R: GAGTTGGGGGACAGCCGAGC	138	Primer-BLAST
CRHR1	F: GCAGCCCGTGTGAATTATTCT R: ATGACGGCAATGTGGTAGTGC	83	Primer Bank
CRHR2	F: TCATCGCCTGGGCAGTTGGC R: CGGACGTGGTGGATGCTCGT	186	Primer-BLAST
MR	F: AATGGTGGGGCCTTGCGTGC R: AGACGGCATGTTGAGCGGGC	90	Primer-BLAST
GR	F: ACCAGCCGTCCAGAGAACCCC R: TCACACTGCCACCGTTGGTGC	148	Primer-BLAST
AVP	F: TCGCCAGGATGCTCAACAC R: TTGGTCCGAAGCAGCGTC	174	Zhang et al. (2011)
V1b	F: CTCTGCCGGGCTGTCAAATA R: TCATGGCCAGCAGCATGTAA	70	Primer-BLAST

Table 6.1: Gene specific forward (F) and reverse (R) primers used for both one step RT-PCR and real-time RT-PCR. Primers were either derived from published sequences, from Primer-BLAST (Basic Local Alignment Search Tool, (Ye et al., 2012)) or from PrimerBank (Spandidos et al., 2010).

6.3 Results

6.3.1 The expression profile of genes of interest in the mouse brain

Reverse transcription PCR (RT-PCR) was used to determine the presence or absence of genes of interest (AVP, CRH, CRHR1, CRHR2, GR, MR and V1b) in the hypothalamus, hippocampus, pituitary gland and PFC of the mouse brain (Figure 6.1). Electrophoresis of RT-PCR products confirmed the presence of a single amplicon of the predicted size for each primer pair tested.

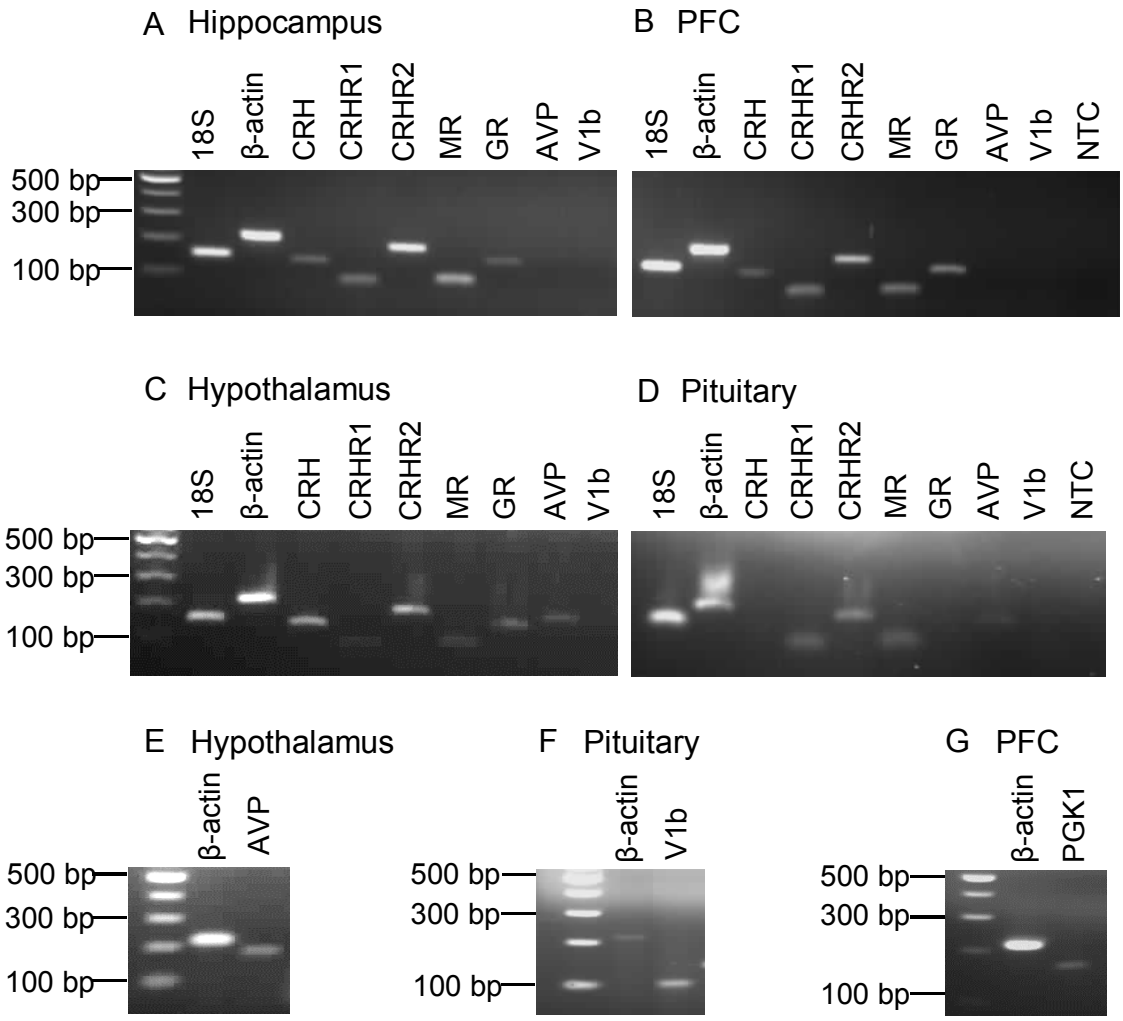

Figure 6.1: Expression of 18S, β-actin, AVP, CRH, CRHR1, CRHR2, GR, MR and V1b in the hippocampus (A), PFC (B), hypothalamus (C) and pituitary gland (D) of the adult BALB/c mouse brain. Further experiments confirmed expression of AVP in the hypothalamus (E), V1b in the pituitary gland (F) and PGK1 in the PFC (G). NTC: no template control.

6.3.2 Selection of a suitable reference gene for qPCR

It is important that the expression of any reference gene used for qPCR is stable across different experimental conditions (Bustin et al., 2009). Initial experiments aimed to compare the stability of three reference genes, 18S ribosomal RNA (18S), β-actin, and PGK1 in adult and juvenile mice, across different brain regions. Using both GeNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) softwares. In all brain regions, expression of PGK1 was found to be more stable in adult and juvenile mice than either 18S or β-actin (Table 6.2). This is consistent with a previous study examining PGK1 gene expression during mouse brain development (Boda et al., 2009). Therefore PGK1 was used as the reference gene for all further qPCR experiments in this thesis.

Rank	Hypothalamus	Pituitary Gland	Hippocampus	Prefrontal Cortex
1st	PGK1	PGK1	PGK1	PGK1 & β-actin
2nd	18S	β-actin	β-actin	18S
3rd	β-actin	18S	18S	

Table 6.2: Expression of PGK1, 18S and β-actin, ranked according to the stability of expression in adult and juvenile mice, as determined by GeNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004) softwares.

In all qPCR experiments, the amplification curve allowed calculation of Cq in order to determine relative changes in gene expression. Figure 6.2 shows a representative amplification curve for a qPCR experiment using PGK1 and AVP primers in adult BALB/c mice. Melt curve analysis was also performed to confirm the amplification of a single product. This is important as SYBR green was used as the reporter fluorescence signal in these experiments and it binds to all double stranded DNA. Figure 6.3 shows representative melt curves for all primers, demonstrating specific amplification of the PCR product as shown by the presence of a single peak.

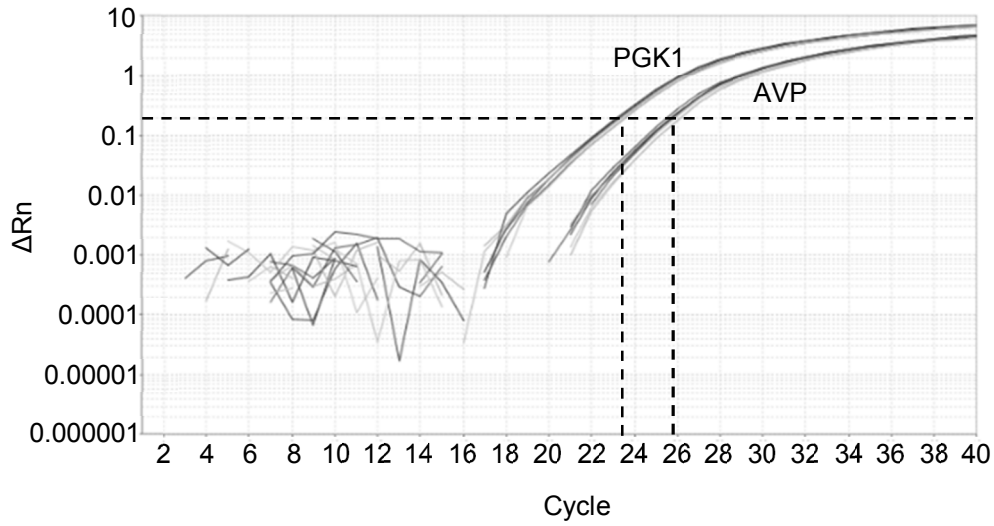

Figure 6.2: Representative qPCR amplification curves using PGK1 and AVP primers in adult BALB/c mice. The quantification cycle (Cq) is the cycle number at which the amplification is exponential and is above a baseline signal (see dotted line) and relative changes in gene expression can be calculated.

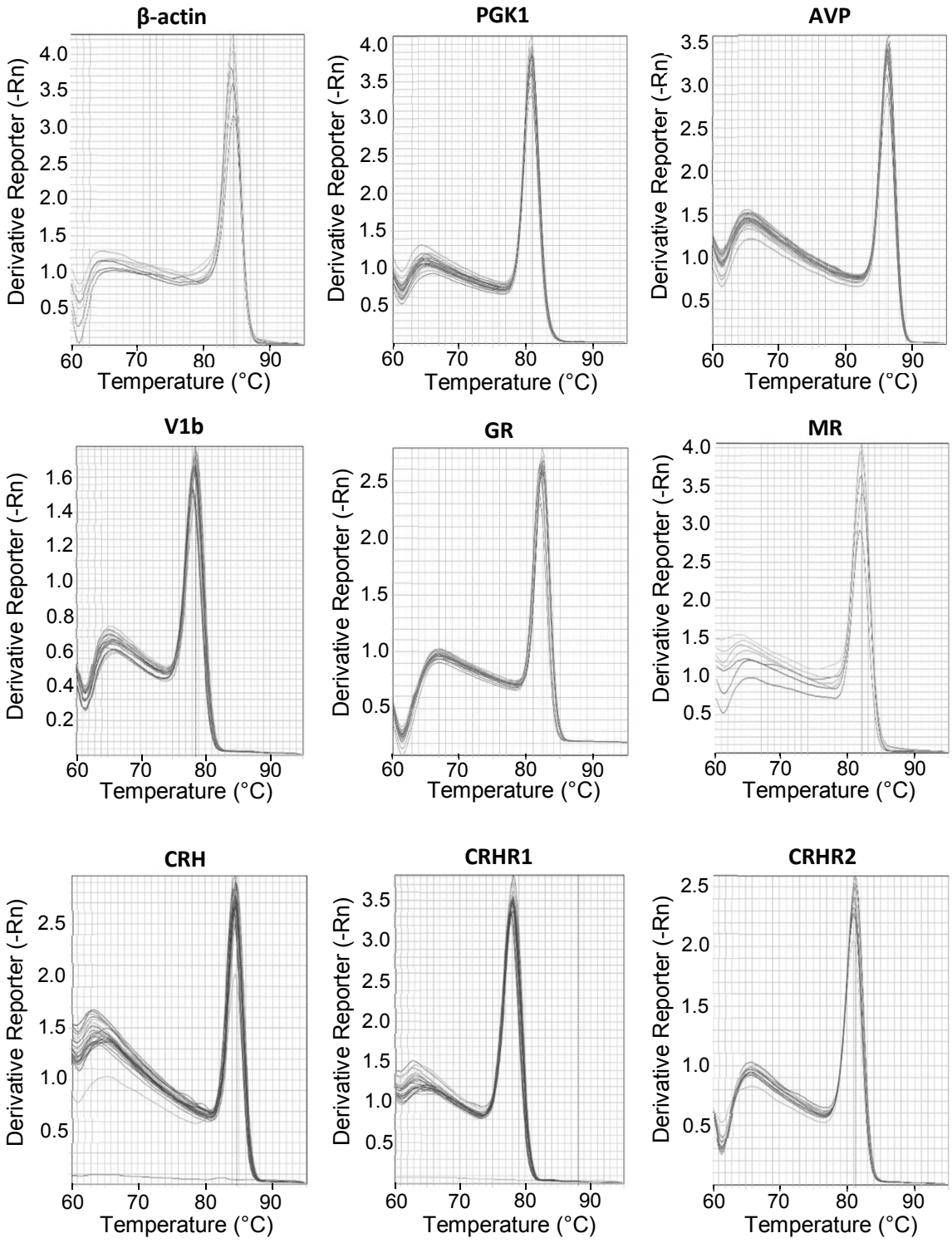

Figure 6.3: Representative melt curves for each primer used, indicating the specificity of the amplicon formed (by the presence of a single peak).

6.3.3 Differences in gene expression in juvenile and adult mice

Initial experiments aimed to determine baseline differences in gene expression in juvenile and adult mice. Naïve mice were handled in the same way as experimental mice, and were killed on the equivalent of experimental day 0, when adult mice were 9-10 weeks old and juvenile mice were 4-5 weeks old. mRNA expression of components of HPA signalling were determined in the hypothalamus, pituitary gland, hippocampus and PFC.

In the hypothalamus, unpaired t-tests showed that AVP expression was significantly higher in juvenile mice than in adult mice ($P<0.05$). CRHR1 expression was significantly lower in juvenile mice than adult mice ($P<0.01$). There was no difference in expression of CRH, CRHR2, GR or MR between adults and juveniles (Figure 6.4).

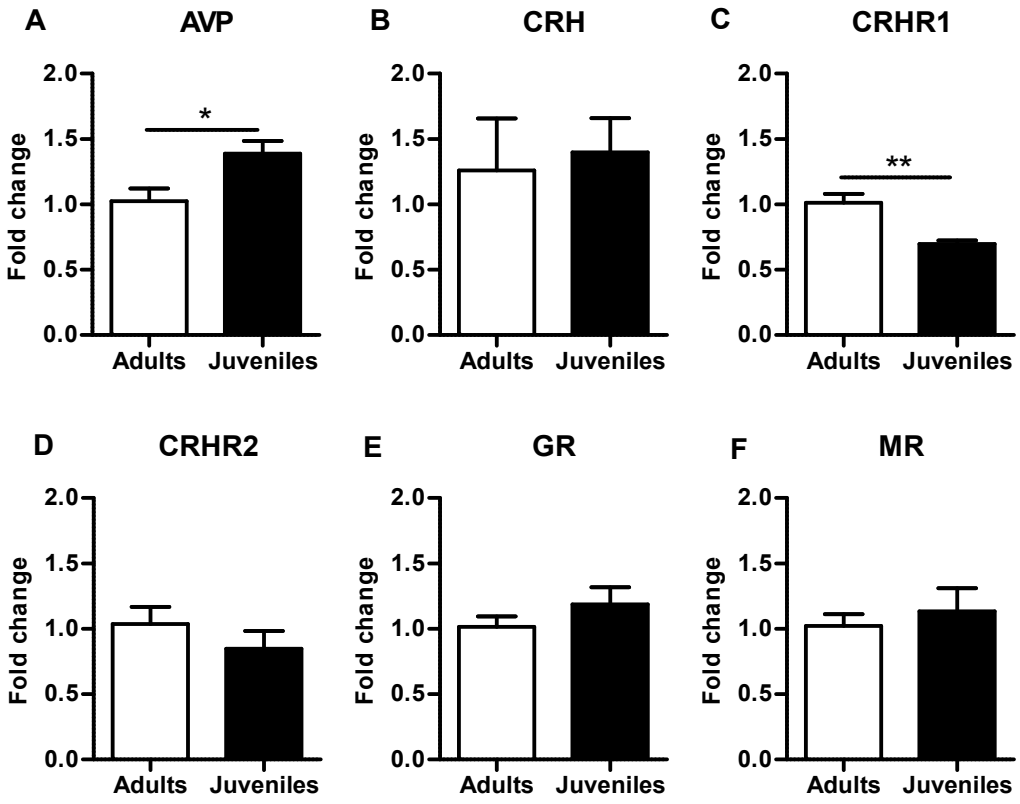

Figure 6.4: Comparison of the expression of AVP, CRH, CRHR1, CRHR2, MR and GR in the hypothalamus of naïve adult and juvenile BALB/c mice. Fold changes are relative to adult mice and normalised to the reference gene PGK1. Results expressed as mean ± SEM, n=6/group. *P<0.05, **P<0.01 (unpaired t-tests).

In the pituitary gland, unpaired t-tests showed that there was no difference in expression of AVP, CRHR1, GR, MR or V1b between adult and juvenile mice (Figure 6.5). Expression of CRH and CRHR2 in the pituitary gland was too low to be quantified.

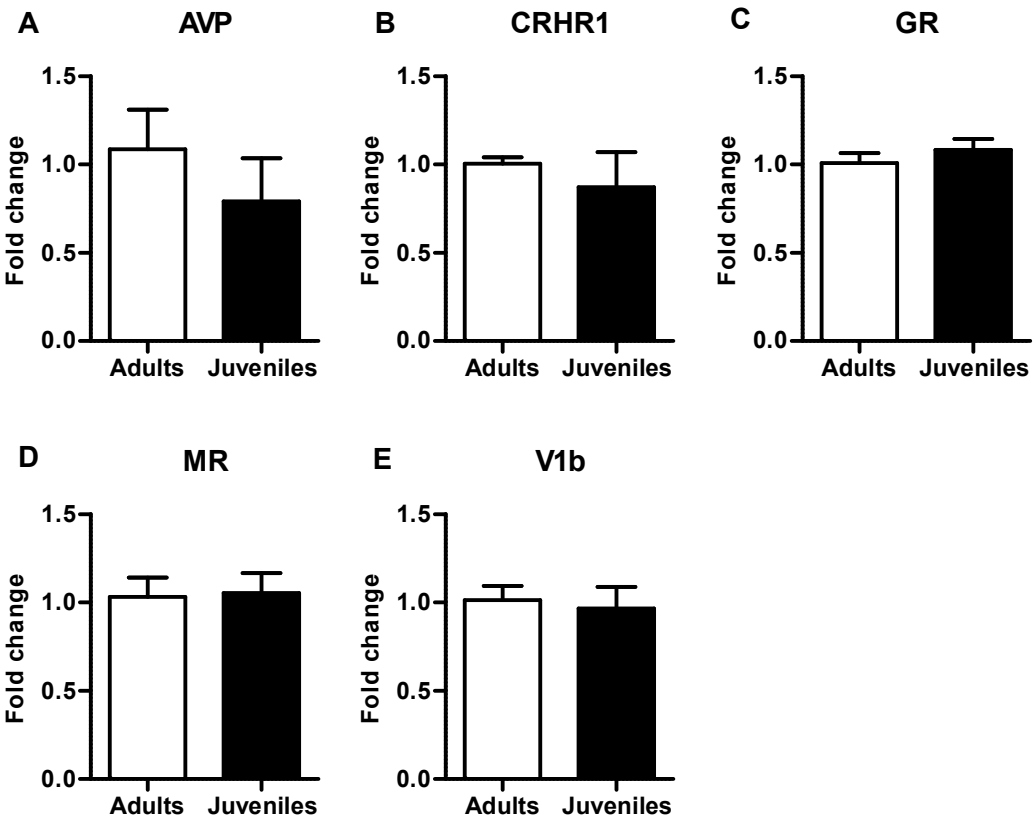

Figure 6.5: Comparison of the expression of AVP, CRHR1, MR, GR and V1b in the pituitary gland of naïve adult and juvenile BALB/c mice. Fold changes are relative to adult mice and normalised to the reference gene PGK1. Results expressed as mean ± SEM, n=5-6/group.

In the hippocampus, unpaired t-tests showed that CRH expression was significantly higher in juvenile mice than in adult mice (P<0.001). There was no significant difference in expression of AVP, CRHR1, CRHR2, GR or MR between adults and juveniles (Figure 6.6).

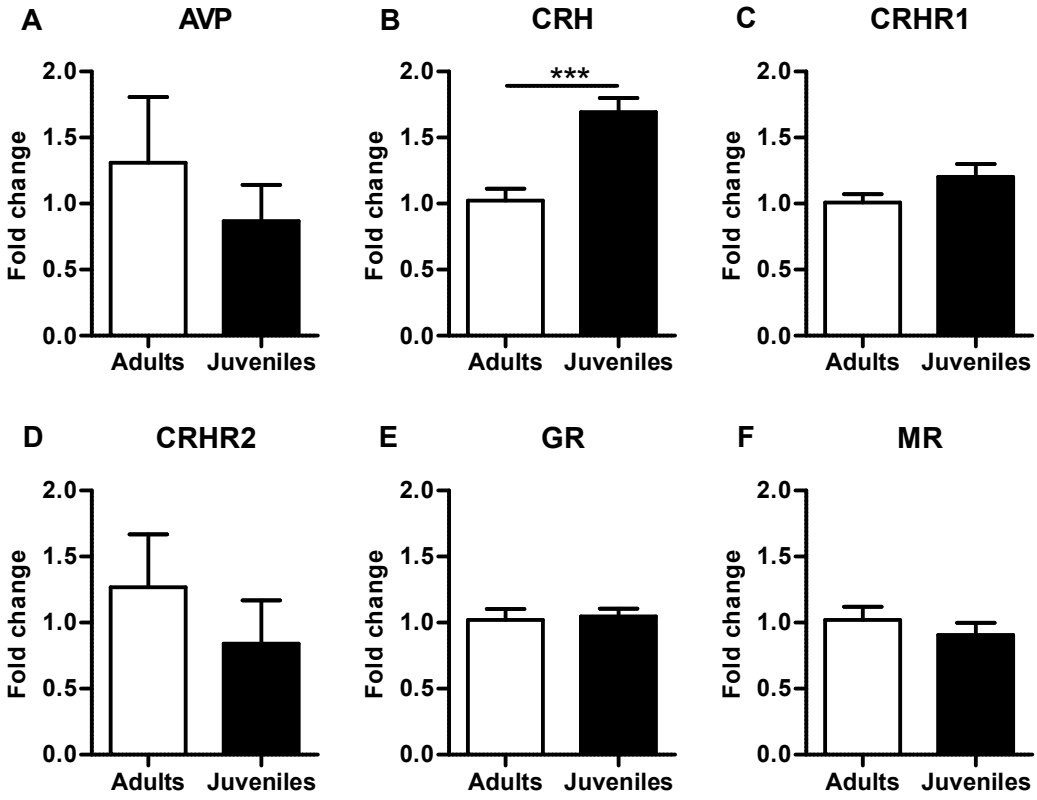

Figure 6.6: Comparison of the expression of AVP, CRH, CRHR1, CRHR2, MR and GR in the hippocampus of naïve adult and juvenile BALB/c mice. Fold changes are relative to adult mice and normalised to the reference gene PGK1. Results expressed as mean±SEM, n=6/group. ***P<0.001 (unpaired t-tests)

In the PFC, unpaired t-tests showed that there was no significant difference in expression of AVP, CRH, CRHR1, CRHR2, GR or MR between adult and juvenile mice (Figure 6.7).

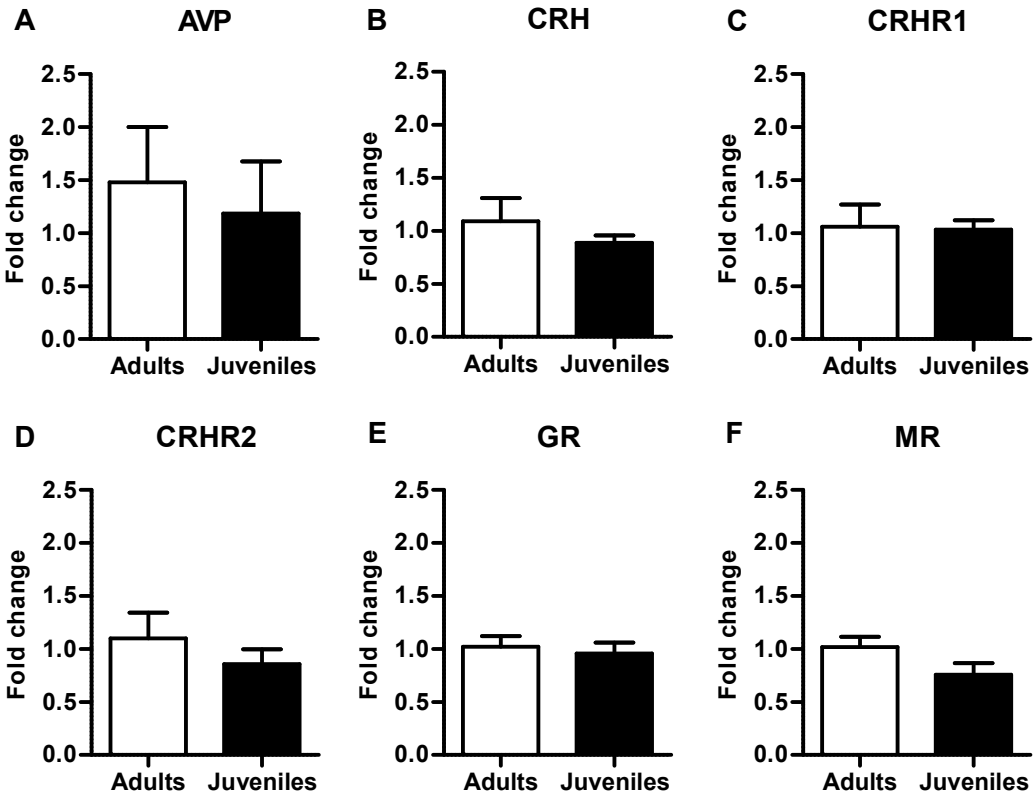

Figure 6.7: Comparison of the expression of AVP, CRH, CRHR1, CRHR2, MR and GR in the prefrontal cortex of naïve adult and juvenile BALB/c mice. Fold changes are relative to adult mice and normalised to the reference gene PGK1. Results expressed as mean ± SEM, n=5-6/group.

6.3.4 Effects of stress on expression of HPA components

To assess the effects of repeated restraint stress on gene expression of components of the HPA axis, mice were killed 2 days following either 3, 7 or 14 days restraint stress and gene expression in the hypothalamus and pituitary gland were determined. Due to time constraints the effects of stress on gene expression were not assessed in the PFC and hippocampus.

In the hypothalamus, there was no significant effect of either 3, 7 or 14 days restraint stress on expression of AVP, CRH, CRHR1, GR, or MR ($P>0.1$, Figure 6.8). There was a significant effect of age on expression of GR ($F_{(1,26)}=12.2$, $P=0.002$) and MR ($F_{(1,26)}=11.6$, $P=0.002$) following 14 days restraint, with juvenile control mice showing significantly lower expression of both GR ($P<0.01$) and MR ($P<0.05$) than adult control mice. Following 7 days restraint, there was a significant effect of age on AVP ($F_{(1,27)}=4.3$, $P<0.05$) and MR ($F_{(1,27)}=11.8$, $P=0.002$) expression, with juvenile control mice having significantly lower expression of both AVP and MR than adult control mice ($P<0.05$). Following 3 days restraint, there was a trend towards a significant effect of age on expression of AVP ($F_{(1,26)}=3.0$, $P=0.09$) and GR ($F_{(1,26)}=3.4$, $P=0.08$), with expression of GR found to be significantly lower in juvenile control mice compared with adults (Figure 6.8).

In the pituitary gland, there was no effect of either stress or age on expression of AVP, CRHR1, GR, MR or V1b following 3 days restraint stress ($P>0.1$, Figure 6.9). Following 7 days restraint, there was a significant effect of both stress ($F_{(1,26)}=6.95$, $P=0.01$) and age ($F_{(1,26)}=31.2$, $P<0.0001$) on expression of CRHR1. Juvenile control mice had significantly lower CRHR1 expression than adult control mice ($P<0.01$). 14 days restraint stress had a significant effect on GR expression ($F_{(1,21)}=5.3$, $P=0.03$), although there were no significant pairwise comparisons following multiple corrections (Figure 6.9).

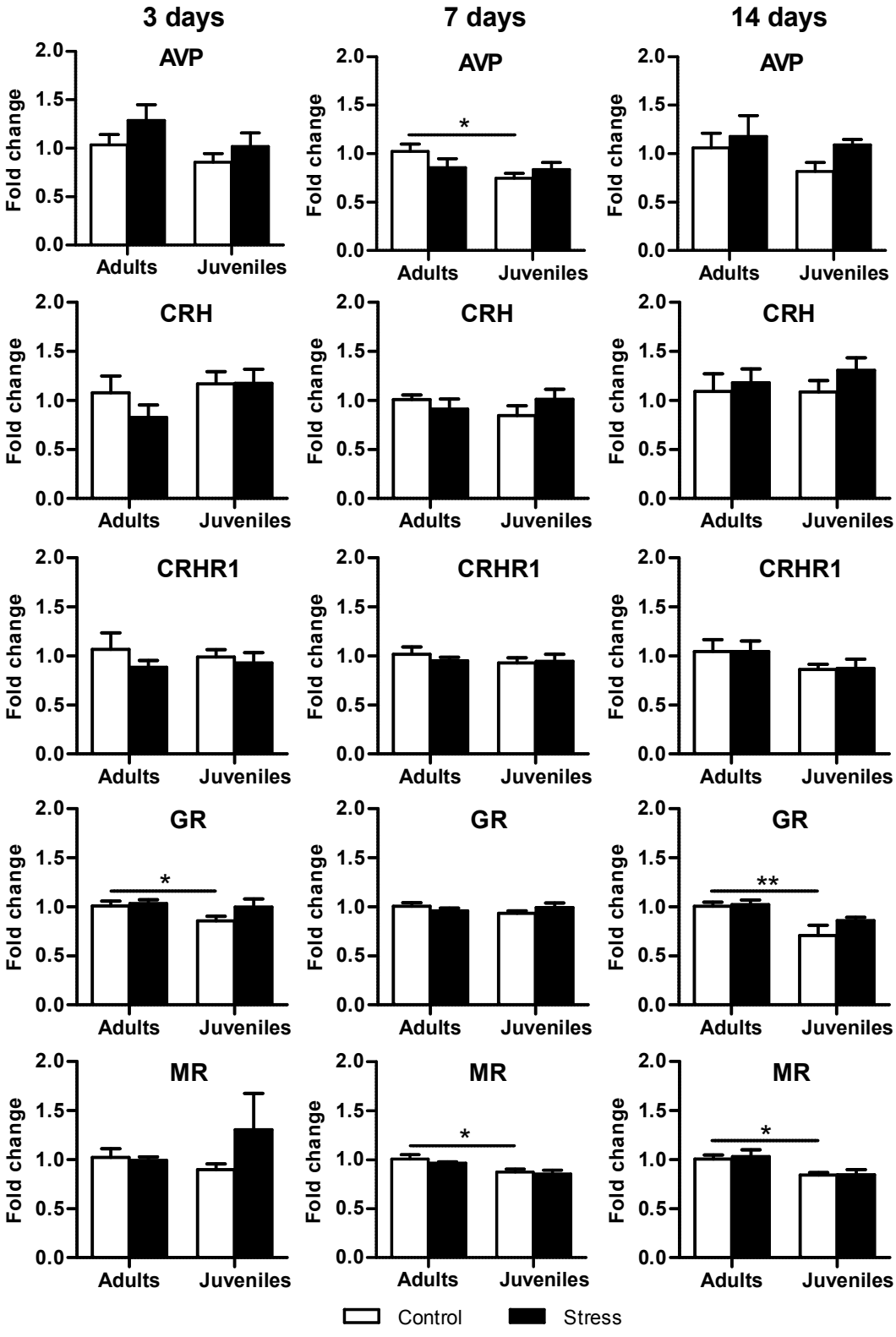

Figure 6.8: Effect of 3, 7 or 14 days stress on expression of AVP, CRH, CRHR1, GR and MR in the hypothalamus of adult and juvenile BALB/c mice. Fold changes are relative to adult control mice and normalised to the reference gene PGK1. Results expressed as mean ± SEM, n=6-8/group (post-hoc LSD test).

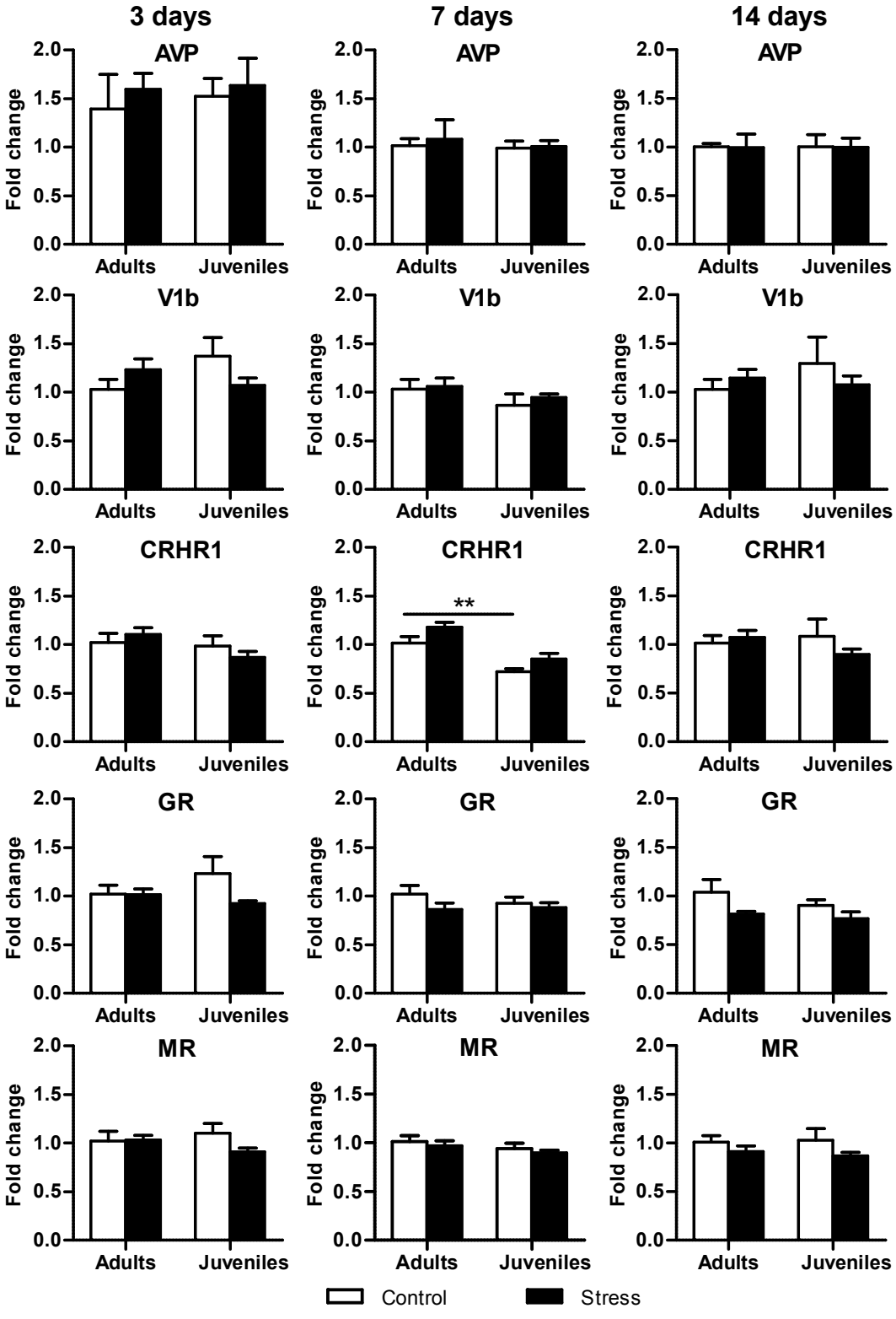

Figure 6.9: Effect of 3, 7 or 14 days stress on expression of AVP, V1b, CRHR1, GR and MR in the pituitary gland of adult and juvenile BALB/c mice. Fold changes are relative to adult control mice and normalised to the reference gene PGK1. Results expressed as mean ± SEM, n=6-8/group (post-hoc LSD test).

6.4 Discussion

The data presented in this chapter are based on qPCR experiments. There are a number of limitations associated with qPCR as set out in the "Minimum Information for publication of Quantitative real-time PCR experiments" (MIQE) guidelines (Bustin et al., 2009). Experimental variability can arise in the tissue sampling, handling, preparation and quality of RNA. In all experiments, control and stressed or adult and juvenile, tissues were collected in parallel, stored and RNA extracted in identical ways. For relative quantification protocols, the use of housekeeping or reference genes as internal controls is an important normalization strategy. Several reference gene mRNAs were investigated for stability of expression across juvenile and adult brains and across multiple brain regions. The widely used 18S ribosomal RNA (18S) and β-actin genes were compared with PGK1 for stability of expression across juvenile and adult brains and across multiple brain regions. In this data set PGK1 expression was found to be stable in adult and juvenile mice in the brain regions of interest. This is consistent with a previous study examining PGK1 gene expression during mouse brain development (Boda et al., 2009). Since SYBR green was used as the detection method it was also essential to confirm that each qPCR reaction yielded a single product of the correct amplicon size. This was confirmed using gel electrophoresis and melting curve analysis. In future studies it would be desirable to confirm the qPCR data at the protein level.

The work in this chapter aimed, for the first time, to determine age-dependent differences in gene expression of components of the HPA axis between juvenile and adult BALB/c mice. Juvenile mice showed a 35% greater expression of AVP, and 30% lower expression of CRHR1 in the hypothalamus, and a 65% greater expression of CRH in the hippocampus, compared with adult mice. Altered sensitivity to secreted hormones of the hypothalamus and pituitary have been reported. For example, lower levels of applied ACTH in preadolescent male rats produced significantly higher corticosterone responses than in adults (Romeo et al., 2014). These authors did not investigate the mechanism

underlying this response. Here, a reduced expression of CRHR1 in juvenile animals could imply a reduced responsiveness to CRH and lower resulting corticosterone responses. However, while the baseline corticosterone response seen in juvenile animals is lower, the response to stress is actually increased (Chapter 4.3). Alternatively, the enhanced stress responsiveness seen in juvenile animals may be mediated by a greater expression of AVP. AVP only weakly stimulates ACTH release on its own, but acts synergistically with CRH to modulate ACTH release (Aguilera and Rabadan-Diehl, 2000, Scott and Dinan, 2002). The actions of AVP, rather than CRH, have been suggested to contribute to the overactivity of the HPA axis during chronic stress (Aguilera and Rabadan-Diehl, 2000). This may also be true in naive juvenile animals compared with adults.

Interestingly, differences in the expression of AVP and CRHR1 in the hypothalamus, seen between naïve juvenile and adult mice, were not apparent between control, non-stressed adult and juvenile mice following the stress studies. Instead, there was lower expression of GR and MR in juvenile control mice compared with adults; differences not seen between naïve juvenile and adult mice. However, these two groups of animals are not directly comparable. The naïve juvenile mice studied here were bought in at 3-4 weeks and used at 4-5 weeks. They were not handled daily and experienced no blood sampling or behavioural experiments prior to brain tissues being removed. The control juvenile mice on the other hand were 4-5 weeks old at the start of the experiment and were therefore older when brains were removed (depending on the duration of the repeated stress), all experienced plasma sampling, sucrose preference behavioural testing and daily weighing. Thus, the experience of these control mice is distinct from the naïve mice and this may account for a distinct pattern of gene expression.

Additionally, this thesis aimed to determine the effect of repeated restraint stress on gene expression of components of the HPA axis in both juvenile and adult BALB/c mice. No significant effects of either 3, 7 or 14 days repeated restraint stress were found on any of the genes examined. This suggests that our hypothesis that gene expression changes in the HPA signalling machinery might underlie the response to stress is not correct. One important component not included in these studies is ACTH and its receptor, the melanocortin 2 receptor. There is emerging data supporting a role for greater adrenal sensitivity to ACTH contributing to the increased corticosterone responses seen in pre-adolescent animals (Romeo et al., 2016). In future studies changes in ACTH release in juvenile and adult mice exposed to repeated restraint stress could be monitored alongside adrenal responsiveness and melanocortin 2 receptor expression.

In this thesis, I have focussed on gene expression of components of the HPA axis. However, it has been shown that other genes are involved in depression. Others have demonstrated higher levels of the $5HT_{1A}$ receptor in mice bred to express more depressive-related behaviour (Kaufman et al., 2016), and noradrenaline transporter knockout mice show reduced depression-related behaviour in the FST (Xu et al., 2000, Czéh et al., 2016). In humans, there have been several genome-wide association studies investigating potential genes involved in depression. While there is evidence that various different genes are associated with depression, including the LHPP gene and the SIRT1 gene (both involved in cellular metabolism), and the serotonin transporter, their potential function in depression is not yet known (Savitz and Drevets, 2009, Hyde et al., 2016, CONVERGE consortium, 2015).

7 General Discussion

Direct comparisons of this study with other reports in the literature are difficult because of differences in restraint stress procedures. It is known that the effects of restraint depend on the duration, frequency and intensity of restraint (Buynitsky and Mostofsky, 2009). Acute restraint stress, a single application, is most commonly used in the literature with rats being studied more often than mice. In comparing restraint paradigms used in adolescent mice a variety of devices have been used, including a modified 50 ml syringe tube as used here (Kim and Han, 2006, Ota et al., 2015), a plastic restraint bag (Uzturk et al., 2015, Jacobson-Pick et al., 2011) or a wire mesh cage (Romeo et al., 2013, Gong et al., 2015). How control animals are handled also varies across laboratories where non-stressed animals may not be handled at all (Jones et al., 1998) or perhaps removed to a novel environment (Ota et al., 2015). Here, the control non-stressed animals were gently handled and weighed daily to ascertain that any behavioural or hormonal effects are due to the restraint stress itself, rather than the handling which is itself a potential stressor (Hurst and West, 2010). This may account for changes in control groups observed in some studies, particularly in the longer duration studies where mice will have been handled for up to 14 successive days. The duration of restraint stress also varies across reports in the literature with some mouse studies using single or repeated restraint stress sessions of 15 min (Jacobson-Pick et al., 2011), 30 min (Romeo et al., 2013), 3h (Ota et al., 2015), 8h (Gong et al., 2015) or longer (Kim and Han, 2006). In this study, we used a 2h restraint which had previously been shown to produce a robust behavioural phenotype in C57BL/6 mice (Kim and Han, 2006). Furthermore, in our pilot studies, a 2h acute restraint stress evoked a significant corticosterone response.

In the literature the timing of behavioural assessments following restraint also varies but behavioural tasks typically are conducted 30-60 minutes post restraint stress in a variety of paradigms. In this study, behaviour was evaluated in a variety of tests including the elevated plus maze, the forced swim test and the sucrose preference test 24-48 hours

post-restraint to assess whether there was lasting impact on the behaviour of the animal from the repeated restraint stress sessions. For example, it has been shown that acute restraint stress in mice does induce anxiety- and depression-related behaviours in the EPM and FST, when behaviour was assessed 20-40 minutes following stress (Freitas et al., 2014, Hsu et al., 2007). In this thesis (Figure 5.10), 24 h following an acute restraint stress, no significant changes in behaviour were seen in the FST, SPT or EPM. This may reflect different strains of mice being used, with Freitas et al. (2014) using Swiss mice, and Hsu et al. (2007) using NMRI mice, compared with the BALB/c mice used here. This timing of behavioural assessment is important in order to distinguish between the acute effects of restraint stress and the long-lasting chronic effects of restraint. This appears to be a novel feature of this experimental design and therefore comparable data in adult and juvenile mice are limited. Thus while this experimental design may preclude the observation of acute behavioural effects following a single restraint stress session (Figure 5.10), it does show that repeated restraint stress and long-lasting behavioural change underlie the adaptive responses seen here.

In humans it has been shown that exposure to stress, particularly during childhood and adolescence, promotes the development of resilience (Lyons et al., 2010, Southwick and Charney, 2012). In addition, heightened responses to stress reactivity in adolescents, compared with adults, have been shown in both mice and humans (Pattwell et al., 2012). Here it is proposed that when juvenile mice are stressed and tested as juveniles, they respond with an adaptive behaviour that may represent a stress coping mechanism. When it was examined whether stress-induced effects in juvenile animals persisted into adulthood (Figure 5.12), there was an apparent increase in open arm entries into the EPM. These data is consistent with the idea that juvenile mice show a greater anxiolytic-like response to stress than adult mice do which may persist into adulthood. This is consistent with previous reports that social crowding stress is anxiolytic in juvenile mice, but not in adults (Ago et al., 2014). Heightened responses to stress reactivity in

adolescents, compared with adults, have also been shown in both mice and humans (Pattwell et al., 2012). The juvenile mice used here were aged 4-6 weeks during exposure to stress and this period corresponds to adolescence in humans (Spear, 2000). It is known that brain areas such as the hippocampus, hypothalamus, PFC and amygdala, key regions involved in the stress response and regulation of emotion and behaviour, continue to develop throughout adolescence and into young adulthood (Spear, 2000).

Together, these findings support the idea that adolescence may be a particularly sensitive period to the effects of stress exposure (Romeo, 2010, Spear, 2000, Eiland and Romeo, 2013); repeated exposure to a predictable stressor may promote resilience and adaptive stress coping behaviours. While this is contrary to the original aims of this thesis, to develop a model of adolescent depression, these studies do provide a potential model for studying the molecular mechanisms underlying resilience.

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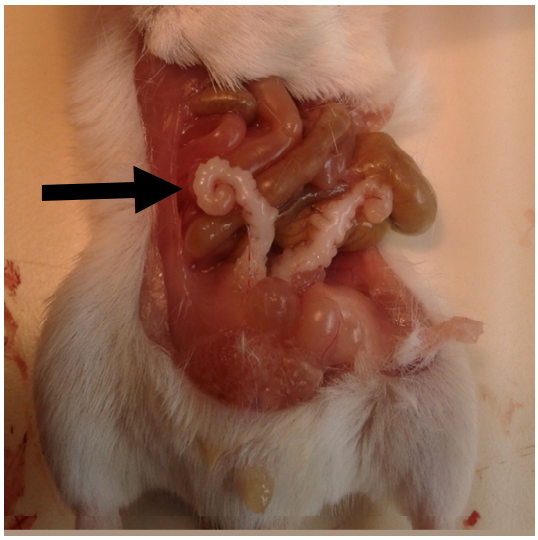
Appendix

Adverse effects of chronic fluoxetine on seminal vesicles in BALB/c mice

In preliminary experiments the effect of chronic fluoxetine was investigated. Adult and juvenile BALB/c mice were treated once daily with fluoxetine 20mg/kg i.p. for up to 28 days. At the end of the experiment, terminal blood samples were collected and adrenal glands were dissected from all mice for analysis of hormone function. In all fluoxetine treated mice, but not saline treated animals, the seminal vesicles looked unusual and apparently fused or knotted into a single blob, rather than the more usual distinctive two separate curling structures (Figure A-1).

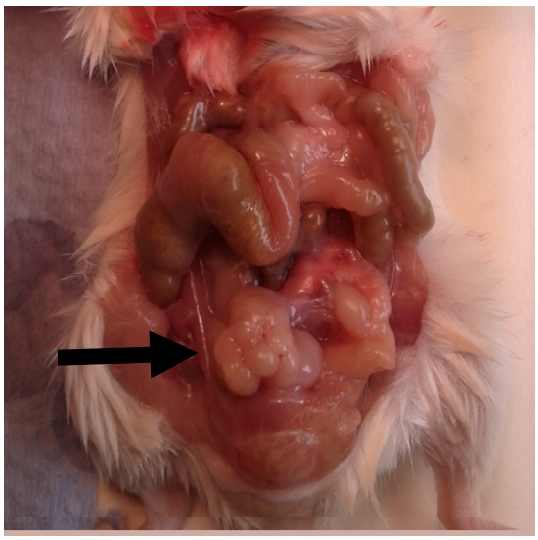
No welfare issues were noted during the chronic treatment period, the fluoxetine treated animals appeared essentially normal. The starting weights of both adult and juvenile animals were the same in saline and fluoxetine treated groups (adult saline: 25.7g ± 1.0g; adult fluoxetine: 25.2g ± 0.5g; juvenile saline: 13.1g ± 0.8g; juvenile fluoxetine: 13.8g ± 0.9g, mean ± S.D., n=8 per group). All animals gained weight during the experiment, were weighed regularly for correct dosing and were weighed at the end (adult saline: 29.7g ± 1.2g; adult fluoxetine: 28.4g ± 1.0g; juvenile saline: 26.2g ± 1.0g; juvenile fluoxetine: 24.9g ± 1.0g, mean ± S.D., n=8 per group). The pH of the fluoxetine solution was approximately 6.5. Histopathological studies of the seminal vesicles revealed no pathological changes in the seminal vesicles associated with fluoxetine treatment, however there was some oedema noted in the surrounding fibrous tissue. This is not a well-described phenomenon in the literature, although one report (Bataineh and Daradka, 2007) has shown that fluoxetine caused seminal vesicle contraction and decreased size in adult male rats, albeit at large doses (200mg/kg “long term fluoxetine diet”).

A: Saline

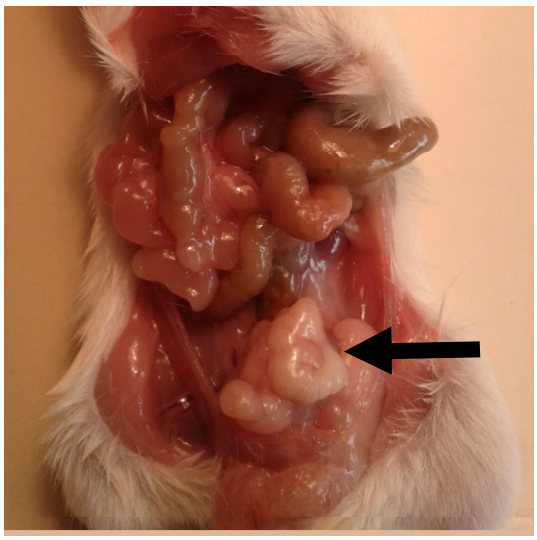


B: Fluoxetine

C: Fluoxetine



D: Fluoxetine



Appendix A-1: Images of seminal vesicles of BALB/c mice following 28 days i.p. treatment with either saline (A) or 20mg/kg fluoxetine (B,C,D). Arrows indicate the seminal vesicles.

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Sadler, A. M. and Bailey, S. J., 2015. Chronic restraint stress increases depression-related behaviour in the sucrose preference test in juvenile and adult mice. *Journal of Psychopharmacology*, 29 (8), A02.

Sadler, A. M. and Bailey, S. J., 2014. Evaluating gender differences in behaviour in the elevated plus maze in CD-1 and C57BL/6 mice. *Journal of Psychopharmacology*, 28 (8), A59.

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Investigating depression-related behaviours in juvenile BALB/c mouse substrains

UNIVERSITY OF BATH

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Introduction

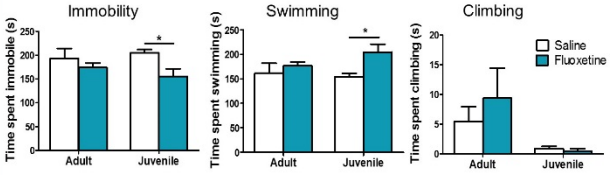
- Adolescent depression is a prevalent condition affecting up to 6% of adolescents (Masi et al, 2000).
- In adolescents, antidepressants used to treat adults are associated with poor clinical benefit and increased suicidal behaviour.
- We aim to develop an animal model of adolescent depression in juvenile mice.
- Here, we have assessed the baseline behaviours, and response to fluoxetine, in juvenile and adult mice of two BALB/c substrains.

Methods

- **Animals**: Male BALB/cJ and BALB/cAnNCrl mice, aged 9-12 weeks (adult) or 5-6 weeks (juvenile) were randomised into either saline control or fluoxetine treatment groups (n=5-8/group).
- **Drug treatment**: Fluoxetine hydrochloride was administered i.p. at a dose of 10 or 20mg/kg. For acute studies, mice were injected 30 minutes before behavioural testing. For chronic studies, mice were injected once daily for 28 days.
- **Forced swim test (FST):** Time spent swimming, climbing and immobile during a 6 minute test session (25°C) were determined by video analysis.
- **Novelty-induced hypophagia (NIH):** Mice were trained for 3 days to drink diluted condensed milk under low light conditions (20 lux). On day 4, mice were tested in their home cage and the latency to drink and consumption of milk in 30 minutes were recorded. On day 5, the test was repeated in a novel cage without bedding and bright light (300 lux).

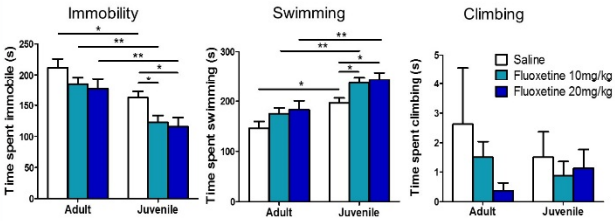
Effects of acute fluoxetine in the forced swim test

BALB/cJ mice



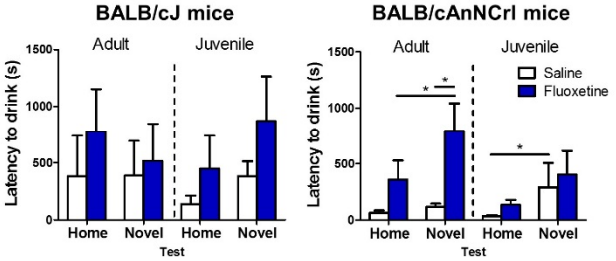
- In BALB/cJ mice, a two-way ANOVA revealed a significant effect of treatment on the time spent immobile ($F_{(1,27)}$=5.66, p<0.05) and swimming ($F_{(1,27)}$=5.40, p<0.05).
- Fluoxetine (10mg/kg) significantly reduced the time spent immobile, and increased the time spent swimming, in juvenile but not adult mice (p<0.05).

BALB/cAnNCrl mice



- In BALB/cAnNCrl mice, a two-way ANOVA revealed a significant effect of treatment ($F_{(2,40)}$=5.62, p<0.01) and age ($F_{(1,40)}$=27.40, p<0.001) on the time spent immobile.
- There was also a significant effect of treatment ($F_{(2,40)}$=6.15, p<0.01) and age ($F_{(1,40)}$=28.71, p<0.001) on the time spent swimming.
- Fluoxetine (10 and 20mg/kg) significantly reduced the time spent immobile, and increased the time spent swimming, in juvenile but not adult mice (p<0.05).
- Juvenile mice spent significantly less time immobile, and more time swimming than adult mice given the same treatment (p<0.05 (saline), p<0.01 (fluoxetine).

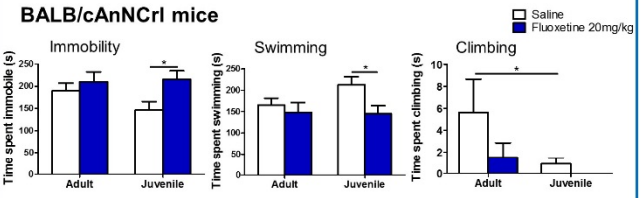
Effects of acute fluoxetine on novelty-induced hypophagia



- In BALB/cJ mice, repeated measures analysis revealed no significant effect of treatment, age or test (home or novel) on latency to drink.
- In BALB/cAnNCrl mice, there was a significant effect of treatment ($F_{(1,28)}$=18.39, p<0.001) and test (home or novel, $F_{(1,28)}$=11.72, p<0.005) on the latency to drink.
- Fluoxetine (20mg/kg) increased the latency to drink in the novel cage compared with saline treated mice (p<0.05) in adult but not juvenile mice.

Effects of chronic fluoxetine in the forced swim test

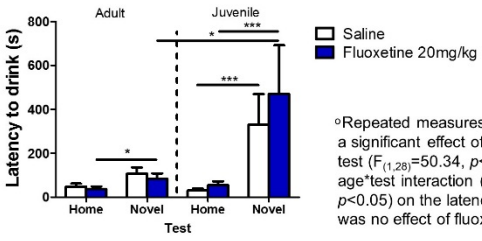
BALB/cAnNCrl mice



- Two-way ANOVA revealed a significant effect of treatment on the time spent immobile ($F_{(1,27)}$=4.99, p<0.05) and swimming ($F_{(1,27)}$=4.58, p<0.05).
- In juvenile (8-9 weeks old), but not adult (13-14 weeks old) mice, fluoxetine *increased* time spent immobile and *decreased* time spent swimming compared to control (p<0.05).

Effects of chronic fluoxetine on novelty-induced hypophagia

BALB/cAnNCrl mice



- Repeated measures analysis revealed a significant effect of home or novel test ($F_{(1,28)}$=50.34, p<0.001) and age*test interaction ($F_{(1,27)}$=7.18, p<0.05) on the latency to drink. There was no effect of fluoxetine treatment.
- Juvenile fluoxetine treated mice had a significantly longer latency to drink in the novel environment compared with adult mice (p<0.05).

Conclusion

- In the FST and NIH, differences in adult and juvenile depression-related behaviour were evident in BALB/cAnNCrl mice but not BALB/cJ mice.
- In juvenile mice, acute fluoxetine displayed an anti-depressant effect in the FST and a pro-depressive effect in the NIH paradigm, whereas chronic fluoxetine exerted a pro-depressive effect in the FST, and no effect on NIH.
- These data suggest that these behavioural paradigms may be suitable for use in a model of adolescent depression using juvenile mice.

References & Acknowledgements

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MRC Medical Research Council

Evaluating gender differences in behaviour in the elevated plus maze in CD-1 and C57BL/6 mice

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Introduction

➢Anxiety disorders are highly prevalent, affecting around 18% of adults in the UK (Fineberg *et al.,* 2013).

➢Anxiety disorders are consistently reported to affect more women than men (Walters *et al*., 2012), however relatively few studies investigating anxiety-related behaviour are conducted using female animals.

➢Genetic variation in different mouse strains may result in differing baseline levels of anxiety-related behaviour between strains (Griebel *et al*., 2000).

➢Here, we have assessed the differences in behaviour in the elevated plus maze in male and female CD-1 and C57BL/6 mice.

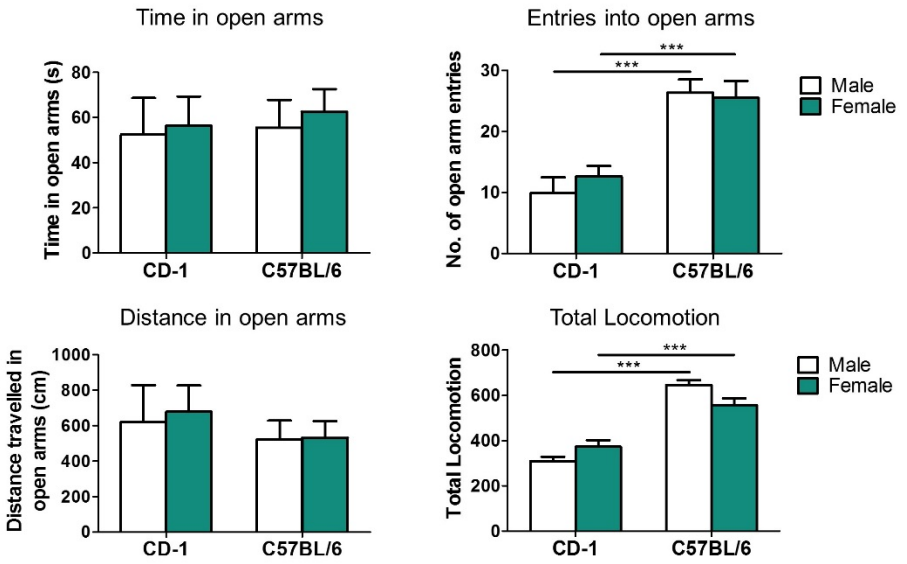
Methods

➢ **Animals:** Male and female CD-1 and C57BL/6 mice (University of Bath), aged 9-10 weeks, were housed in gender groups of 3-4 (n=10/group. Data expressed as mean±SEM).

➢ **Elevated Plus Maze:** Mice were tested in the elevated plus maze (EPM, Campden Instruments) in a 5 min session under lighting conditions of 150 lux on the ends of the open arms, 20 lux in the centre of the maze and <1 lux in the closed arms. Behaviour in the EPM was recorded by MotorMonitor software using infrared beam breaks.

➢**Statistics:** Data were analysed with a 2-way ANOVA and post-hoc LSD test, with Bonferroni's correction for multiple comparisons (InVivoStat software).

Results

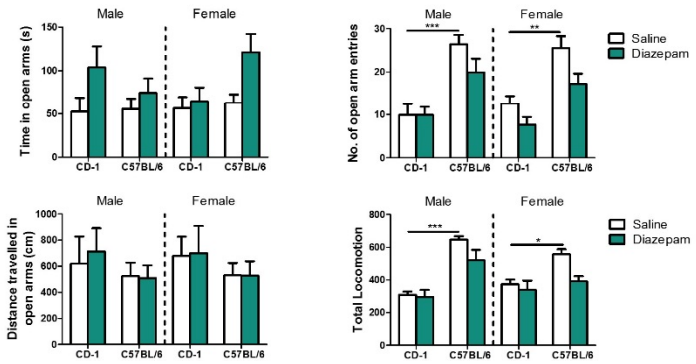


➢There was no effect of sex or strain, or sex*strain interaction, on the time spent in the open arms, or the distance travelled in the open arms.

➢There was a significant effect of strain on the number of open arm entries ($F_{(1,36)}$=0.58, P<0.0001). C57BL/6 mice of both sexes made significantly more open arm entries than CD-1 mice (P<0.001).

➢There was also a significant effect of strain ($F_{(1,36)}$=104.43, P<0.0001) and sex*strain interaction ($F_{(1,36)}$=9.09, P<0.005) on the total locomotion over the whole maze. Again, in both male and female mice, C57BL/6 mice displayed significantly higher locomotor activity than the CD-1 strain (P<0.001).

Results



➢Mice were administered with either diazepam (2mg/kg) or saline (0.9%) i.p. 30 minutes before testing in the EPM.

➢The was a significant effect of diazepam treatment on the time spent in the open arms ($F_{(1,67)}$=8.6, P<0.005), number of open arm entries ($F_{(1,67)}$=8.52, P<0.005) and total locomotion ($F_{(1,67)}$=8.26, P<0.01), but in all cases post-analysis revealed no significant effect of diazepam following correction for multiple comparisons.

➢This may be because the study was underpowered. We are currently conducting further experiments to determine the effects of diazepam.

Conclusions

➢These data show that C57BL/6 mice of both sexes exhibit higher locomotor activity than CD-1 mice.

➢There were no gender differences in anxiety-related behaviour in either strain, which is consistent with other work in both C57BL/6 and CD-1 mice (Frick *et al*., 2000, Parra *et al*., 2002).

➢There remains some controversy over the effect of the oestrus cycle on anxiety-related behaviour. In this study we have not accounted for differing phases of oestrus although we are currently investigating how it may influence behaviour in the EPM and response to benzodiazepines.

References & Acknowledgements

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Fineberg N, Haddad P, Carpenter L, Gannon B, Sharpe R, Young A, Joyce, E, Rowe J, Wellsted D, Nutt D and Sahakian B (2013). The size, burden and cost of disorders of the brain in the UK. *Journal of Psychopharmacology* **27**(9): 761-770

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Published papers

Sadler, A.M. and Bailey, S. J., 2016. Repeated daily restraint stress induces adaptive behavioural changes in both adult and juvenile mice. *Physiology and Behavior,* 167 pp. 313-323.

Sadler, A. M. and Bailey, S. J., 2013. Validation of a refined technique for taking repeated blood samples from juvenile and adult mice. *Laboratory Animals*, 47 (4), pp. 316-319.

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Repeated daily restraint stress induces adaptive behavioural changes in both adult and juvenile mice

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HIGHLIGHTS

- Repeated daily restraint stress in mice induced a complex behavioural adaptation of anxiolytic-like and anhedonic responses
- Juvenile mice showed a wider range of behavioural responses following repeated daily restraint stress than adults
- Novel features of the methodology show behavioural changes result from lasting, not acute, effects of restraint stress
- Behavioural responses to repeated restraint stress were qualitatively similar in C57BL/6 and "stress-sensitive" BALB/c mice
- Adaptive behavioural changes seen may reflect resilience and be beneficial in future stress challenges

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ABSTRACT

Chronic stress is known to be a risk factor for the development of depression and anxiety, disorders which often begin during adolescence. Restraint stress is a commonly used stressor in adult rodents, however the effects of repeated restraint stress in juvenile mice have not been well characterised. Here we have shown for the first time the behavioural and hormonal effects of repeated restraint stress in both adult and juvenile BALB/c and C57BL/6 mice. Repeated daily restraint stress (2 h/day for 3, 7 or 14 days) provoked a robust physiological response evident as increased corticosterone levels and decreased body weight after 14 days. However, habituation of the stress-response was evident during repeated exposure to the stressor in both adult and juvenile mice. The behavioural changes seen in response to repeated restraint stress were complex. In juvenile mice, repeated restraint stress evoked an increase in exploratory behaviours in the elevated plus maze, a decrease in time spent immobile in the forced swim test and a decrease in sucrose preference. In adult mice fewer behavioural changes were seen. Interestingly BALB/c and C57BL/6 mice showed qualitatively similar response to 3 days repeated restraint stress. The behavioural changes we observed, as a result of prior stress exposure, may represent an adaptive stress-coping response or resilience. Both the hormonal and behavioural effects of stress were more pronounced in juvenile mice than in adults. This wider range of behavioural responses seen in juvenile mice might reflect a greater ability to engage in adaptive stress-coping strategies that likely have beneficial effects evident in future stress challenges.

1. Introduction

Chronic stress is known to be a major risk factor for the development of many psychiatric disorders, including depression [1–3]. During adolescence, brain development and physical changes associated with puberty are thought to make individuals particularly vulnerable to the effects of stress [4–6]. There is also increasing evidence that the onset of many psychiatric disorders occurs during adolescence, with up to half of adult disorders having begun by the teenage years [7,8].

Changes in the function of the hypothalamic-pituitary-adrenal (HPA) axis also occur during adolescence [9]. In humans, levels of basal cortisol increase with age and pubertal development throughout the adolescent years [9–11]. This pattern has also been shown in rodents, with an increase in corticosterone from birth through to adulthood [12,13]. Changes in the reactivity of the HPA axis in response to stress have also been reported during adolescence. For example, increases in cortisol release in response to a social stress test have been seen in adolescents over the age of 13 years, compared to those under 13 years old [9]. In rats, chronic restraint stress induces greater increases in corticosterone release in juvenile animals compared with adults [14,15]. These differences in stress-responsiveness between adolescents

Abbreviations: EPM, elevated plus maze; FST, forced swim test; HPA, hypothalamic-pituitary-adrenal; SPT, sucrose preference test.

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0031-9384/

and adults may result in increased vulnerability to the effects of stress during adolescence [13].

Restraint stress is a widely used model of stress in rodents, as it is straightforward to administer, painless and does not cause physical harm to the animals [16]. There have been several studies examining the effects of repeated restraint stress on changes in depression and anxiety-related behaviours. For example, in adult C57BL/6 and NMRI mice, repeated restraint stress has been shown to increase depression and anxiety-related behaviours, in the forced swim test, tail suspension test and elevated plus maze [17–19]. These changes in behaviour have been shown to persist for up to 3 months after the period of stress [20]. In contrast, there are few reports of the behavioural effects of restraint stress in juvenile animals.

Adolescence is a period of vulnerability for the development of depression. Here, we have examined whether repeated restraint stress in juvenile mice induces a behavioural change consistent with an increase in depression-related behaviour. Juvenile mice aged 4–6 weeks are considered to model human adolescence, as at this age they undergo a growth spurt, sexual maturation and developmental brain changes similar to those seen in adolescent humans [5]. In this study, we have investigated the effects of different durations of repeated restraint stress in both adult and juvenile BALB/c mice on depression- and anxiety-related behaviours, coupled with an assessment of HPA function by measuring corticosterone levels. Furthermore, in some studies we have compared both the behavioural and neuroendocrinological effects of stress in BALB/c mice, with those in C57BL/6 mice. It has previously been suggested that BALB/c mice are more sensitive to the effects of stress than the C57BL/6 strain which are relatively stress resilient [21,22].

2. Methods

2.1. Animals

Male BALB/cAnNCrl mice (Charles River UK) and male C57BL/6 mice (University of Bath) were 9–10 weeks old (adults) or 4–5 weeks old (juveniles) at the start of experiments. Mice were either individually housed (BALB/c mice) or housed in groups of 3–4 (C57BL/6 mice) in 35 × 20 × 15 cm polysulfone cages (Plexx, Elst, The Netherlands) with woodchip bedding (Datesand, Manchester, UK) and paper nesting material (Lillico/LBS Biotechnology, Horley, UK). The mice were maintained in a temperature (21 ± 1 °C) and humidity (50–60%) controlled environment, under a 12 h light/dark cycle (lights on 07:00 h), with food and water available ad libitum. All mice were acclimatised to the animal facility for at least 4 days prior to the start of experiments, during which time they were handled on at least two occasions by gentle cupping [23]. Experiments were conducted during the light phase, between 08:00–13:00 h, with the exception of the sucrose preference test which occurred during the dark phase. All procedures were carried out under a Home Office project licence held in accordance with the Animals (Scientific Procedures) Act 1986.

2.2. Restraint stress

Stressed mice were placed head first into a modified 50 ml syringe with ventilation holes, which was plugged with the syringe plunger and adjusted so that mice were unable to move forwards or backwards. Mice were restrained for 2 h each day, for either 3, 7 or 14 consecutive days. Stressed mice were weighed daily, and monitored for signs of distress using a scoring system adapted from Lloyd and Wolfensohn [24] (Supplementary Fig. 1). Non-stressed control mice were weighed daily, but otherwise remained in their home cage.

2.3. Behavioural testing

Behavioural testing in the elevated plus maze and forced swim test occurred in a dimly lit room adjacent to the animal holding room. Mice were left to acclimatise in the behavioural room for at least 1 h prior to testing. Testing in the elevated plus maze, forced swim test and sucrose preference test occurred one day following 3 days restraint, or two days following 7 or 14 days restraint. Separate groups of mice were used for each behavioural test. A diagram outlining the restraint stress protocol is shown in Fig. 1.

2.4. Forced swim test (FST)

Mice were placed in a glass beaker (diameter 22 cm, height 34 cm), filled to a depth of 23 cm with water (25 °C). Each 6 min test session was filmed and behaviour in the last 4 min of the test was scored by an experimenter blind to treatment. Swimming was defined as horizontal movement, and immobility defined as the minimal activity needed to stay afloat. Following the test session, mice were dried with paper towels and placed in a warm holding cage, before being returned to the home cage. The water was replaced, and the beaker cleaned with 70% ethanol, between each mouse.

2.5. Sucrose preference test (SPT)

On the last day of restraint, mice were habituated to drinking from 2 bottles of water, for 12 h (19:00–07:00 h). The following day, mice were given the choice to drink either water or 5% w/v sucrose (BALB/c mice), or water and 2.5% w/v sucrose (C57BL/6 mice) during a 12 h test (19:00–07:00 h). Inbred mouse strains are known to have differing sensitivities to sucrose [25], so the concentration of sucrose required to produce preference was determined in preliminary experiments (Supplementary Fig. 4). Bottles were weighed before and after the test, and the preference for sucrose was determined as a percentage of the total volume consumed. Total volume consumed was also determined.

2.6. Elevated plus maze (EPM)

The elevated plus maze (Campden Instruments) consisted of four arms (38 × 5 cm), arranged at right angles around a central intersection (5 × 5 cm). Two of the arms were open, with a 0.5 cm rim, while the other two were enclosed with 15 cm high walls. The entire maze was elevated 65 cm off the floor. Lighting on the open arms measured 20 lx for experiments with BALB/c mice, and 50 lx for experiments using C57BL/6 mice. Mice were placed in the central intersection facing an open arm and allowed to freely explore the maze for 5 min. Time spent in, and number of entries into the open arms, and total locomotion over the whole maze, were recorded by MotorMonitor™ software using infrared photobeams. The maze was cleaned with 70% ethanol after each mouse.

2.7. Assessment of neuroendocrinological function

Blood samples were taken from all mice at baseline and immediately following the last restraint stress. 40 μl samples were taken from the lateral tail vein [26] and collected in heparinised capillary tubes (Hawksley, Sussex, UK). Blood was transferred to micro-centrifuge tubes containing EDTA (final concentration in sample 3 μg/μl) and kept on ice until being centrifuged at 2000 rcf for 20 min at 4 °C. Plasma was removed and stored at −20 °C until analysis. The concentration of corticosterone in plasma was determined using an ELISA (IBL International, Hamburg, Germany). All blood samples were taken between 11:00–13:00 h. To assess the effect of repeated restraint stress on adrenal gland weight, mice were killed by cervical dislocation two days after the last session of restraint, and adrenal glands were removed and weighed. Function of negative feedback inhibition of the HPA axis was determined using the dexamethasone suppression test (DST), which has been used extensively to indicate abnormalities in negative feedback of the HPA axis in depressed patients [27]. A baseline blood sample was taken between 3 and 4 pm, on the day after the last session of

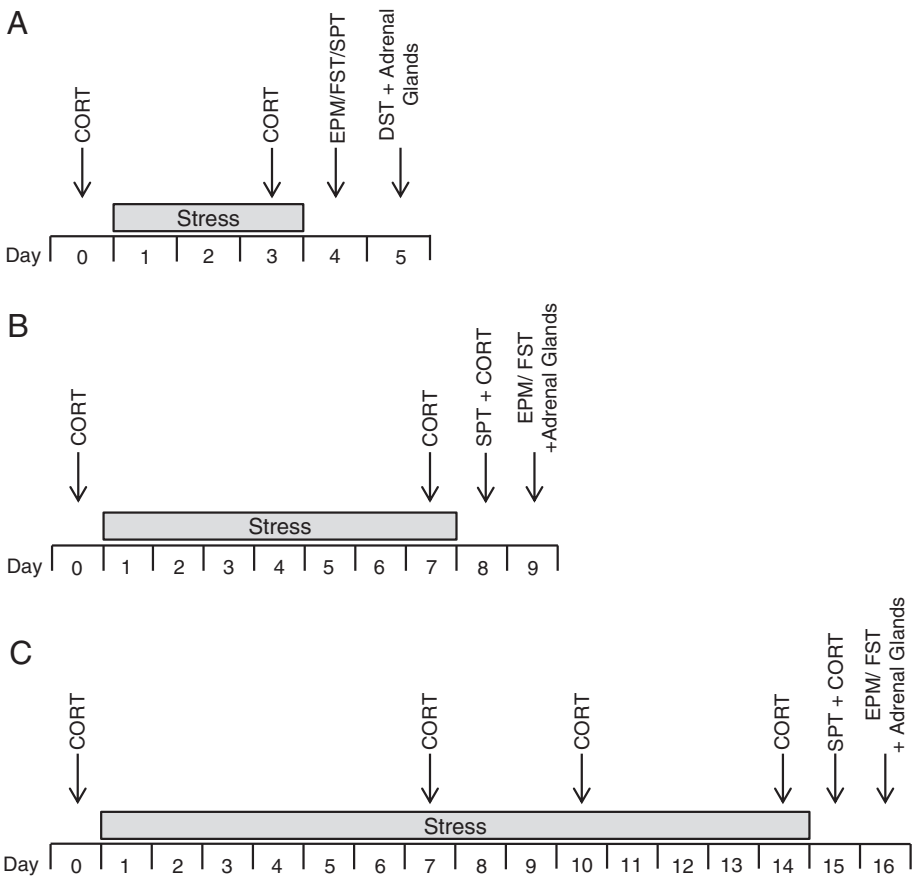

Fig. 1. Experimental design for mice undergoing either 3, 7 or 14 days restraint stress. CORT, blood taken for corticosterone analysis; DST, dexamethasone suppression test; EPM, elevated plus maze; FST, forced swim test; SPT, sucrose preference test.

restraint. The DST occurred 2 days after stress, when mice were administered 0.1 mg/kg dexamethasone (dexamethasone 21-phosphate disodium, (Alfa Aesar), 10 ml/kg, i.p., dissolved in 0.9% w/v saline) between 9 and 10 am, and had a blood sample taken 6 h later to determine corticosterone-induced suppression of corticosterone release. Blood samples were processed and corticosterone levels in plasma determined as described above.

2.8. Statistical analysis

All statistical analysis was performed using InVivoStat software Version 2.5.0.0 [28]. Elevated plus maze and forced swim test data were analysed using unpaired *t*-tests to compare the effects of stress with non-stressed control mice. Adrenal gland weights in BALB/c mice were analysed by a two-way ANOVA with post hoc least significant difference (LSD) test for planned pairwise comparisons followed by Bonferroni's correction for multiple comparisons (Fig. 3). Adrenal gland data in C57BL/6 mice were analysed using unpaired *t*-tests to compare the effects of stress with non-stressed control mice because of a different experimental design (Fig. 8). Sucrose preference tests were analysed using two-way ANOVA, with age and stress as factors. LSD test with Bonferonni's correction for multiple comparisons was used for post-hoc comparisons. Corticosterone and DST data were analysed using repeated measures mixed model analysis followed by pairwise comparisons [28], with Bonferonni's correction for multiple comparisons. The relationship between corticosterone and adrenal gland weight was determined using a Pearson's correlation. For statistical analysis, all corticosterone data, and the majority of behavioural data were $\log_{10}$ transformed to stabilise the variance. All data is presented as mean ± SEM, and significance was taken as $p < 0.05$.

3. Results

3.1. Effects of repeated restraint stress on neuroendocrinological measures

To determine HPA activation in response to restraint stress, blood samples were taken at baseline and following 3, 7 or 14 days restraint, and plasma levels of corticosterone were determined (Fig. 2). At all timepoints, blood samples were also taken from non-stressed control mice for comparison. There were no differences between control and stressed groups in baseline plasma corticosterone measures. After 3 days restraint stress, there was a significant effect of stress on plasma corticosterone in both adult ($F_{(1,29)} = 49.75$, $p < 0.001$) and juvenile BALB/c mice ($F_{(1,27)} = 35.76$, $p < 0.001$), with stress resulting in a 710% increase in corticosterone over baseline in adults, and a 2000% increase in juveniles ($p < 0.001$). Similarly, there was a significant effect of 7 days restraint on corticosterone in both adults ($F_{(1,28)} = 18.17$, $p < 0.001$) and juveniles ($F_{(1,30)} = 13.9$, $p < 0.001$). In a separate group of animals the sequential effects of 7, 10 and 14 days repeated restraint on HPA function was examined in adults (Fig. 2E). For juvenile animals only the effects of 7 and 14 days repeated restraint were compared because of limitations with blood volumes (Fig. 2F). A significant increase in plasma corticosterone was evident at 14 days in both adult ($F_{(1,29)} = 69.86$, $p < 0.001$) and juvenile mice ($F_{(1,14)} = 10.49$, $p < 0.01$), compared to handled, non-stressed control mice. However, at 14 days, there was no significant difference in corticosterone responses in adult stressed mice measured at baseline (pre-stress) and after 14 days of repeated restraint stress, and the corticosterone response appeared attenuated compared with 7 days restraint. The elevation in corticosterone levels in stressed mice did not persist 24 h post-restraint in either adult or juvenile animals.

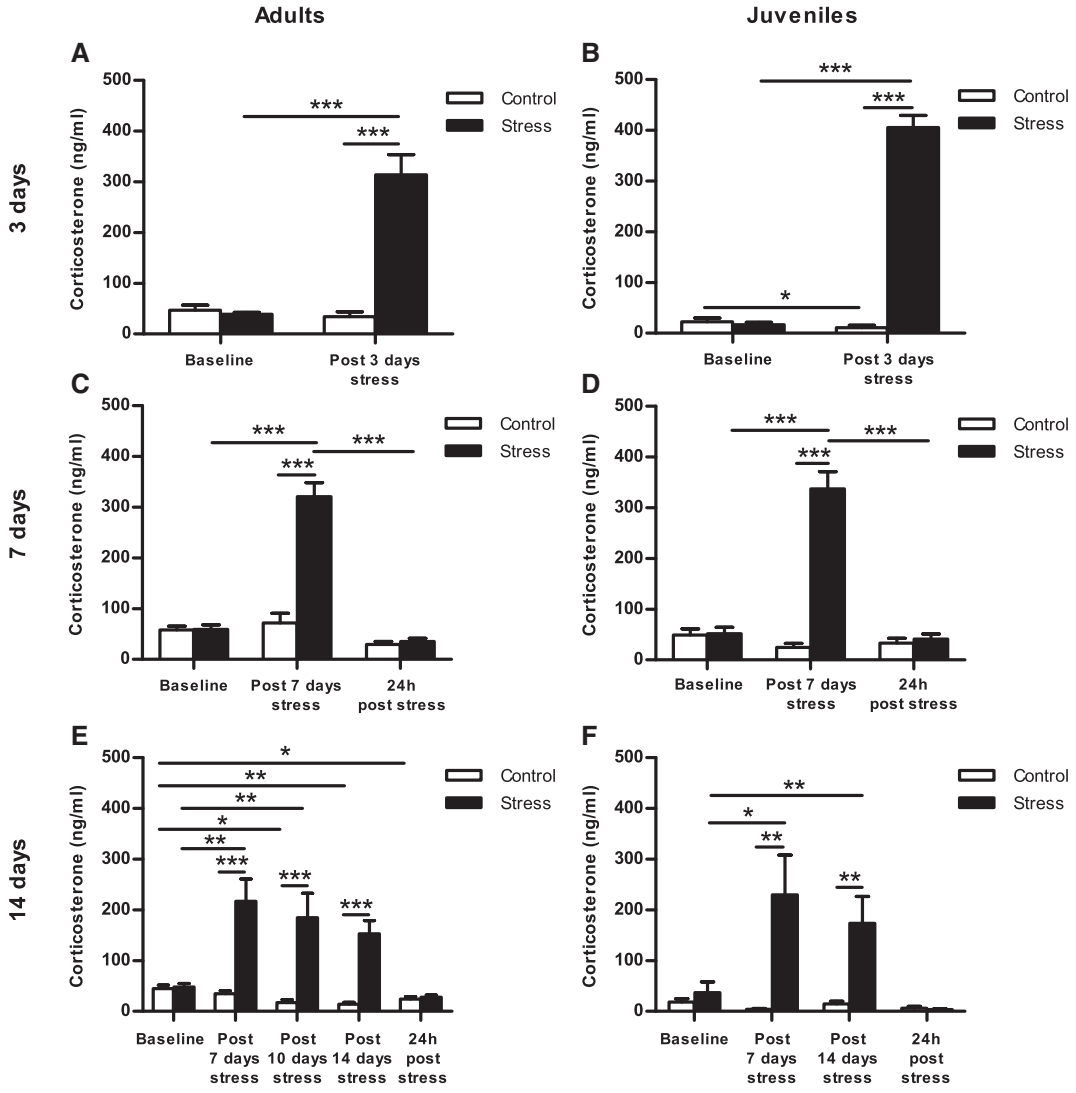

Fig. 2. Effect of 3, 7 or 14 days restraint stress on plasma corticosterone in (A) adult and (B) juvenile BALB/c mice. Blood samples are taken at baseline, immediately following 3, 7 and 14 days restraint, and 24 h following the last session of restraint. All blood samples taken during the light phase 11:00–13:00 h. Results are expressed as mean ± SEM, $n = 4$–16/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (repeated measures mixed model analysis).

Adrenal gland weight, corrected for body weight, also demonstrated that the restraint stress procedure impacted on the HPA axis (Fig. 3). Following 3 days restraint stress, there was a trend towards a significant effect of stress ($F_{(1,25)} = 3.65$, $p < 0.07$), and a significant effect of age ($F_{(1,25)} = 12.23$, $p < 0.01$) on adrenal gland weight. Stress resulted in a 3% increase in adrenal gland weight in adult mice, and a 10% increase in juvenile mice compared with control, although this was not statistically significant. Extending the duration of the restraint stress to 7 days revealed a significant effect of stress ($F_{(1,26)} = 8.66$, $p < 0.01$) and age ($F_{(1,26)} = 19.72$, $p < 0.001$) on adrenal gland weight. There was an 8% increase in adrenal gland weight in adult mice ($p = 0.2$), and a 9% increase in juvenile mice ($p = 0.08$), compared with non-stressed controls. Juvenile control mice had significantly heavier adrenal glands than adult control mice ($p < 0.05$). After 14 days restraint, there was a significant effect of stress ($F_{(1,25)} = 8.55$, $p < 0.01$) and age ($F_{(1,25)} = 23.93$, $p < 0.0001$) on adrenal gland weight. While there was an increase in adrenal gland weight in both stressed adults (9% above control, $p = 0.2$) and juveniles (8% above control, $p = 0.1$), this was not significant following correction for multiple comparisons. Juvenile control mice had significantly heavier adrenal glands than adult control mice ($p < 0.01$).

A Pearson correlation coefficient was calculated to determine whether there was a relationship between corticosterone levels immediately following stress, and adrenal gland weight. There was a positive correlation between corticosterone and adrenal gland weight in both adult ($r = 0.44$, $n = 49$, $p < 0.005$) and juvenile ($r = 0.50$, $n = 46$, $p < 0.001$) mice (Supplementary Fig. 2).

Finally, body weight was recorded daily to assess the effects of repeated stress. At the start of the experiment the mean body weight for adult mice (9–10 weeks) was 26.0 ± 0.5 g (control) and 26.8 ± 0.5 g (stressed), and for juvenile mice (4–5 weeks) was 16.2 ± 0.8 g (control) and 17.4 ± 0.8 g (stressed). Fig. 4 shows the change in body weight, expressed as a percentage of starting body weight, during 14 days repeated restraint stress. In adult mice, there was a significant effect of stress on bodyweight ($F_{(1,14)} = 36.07$, $p < 0.001$), with stressed mice having up to 8% lower bodyweight than controls ($p < 0.01$). Similarly, in juveniles there was a significant effect of stress on bodyweight ($F_{(1,14)} = 8.92$, $p < 0.01$), with stressed mice having up to 12% lower bodyweight than non-stressed control mice ($p < 0.01$). Shorter duration restraint stress had no significant effect on body weight, with adult stressed mice showing a 3% reduction, and juvenile mice showing a 5–10% reduction, after 3 and 7 days restraint (all p's > 0.1).

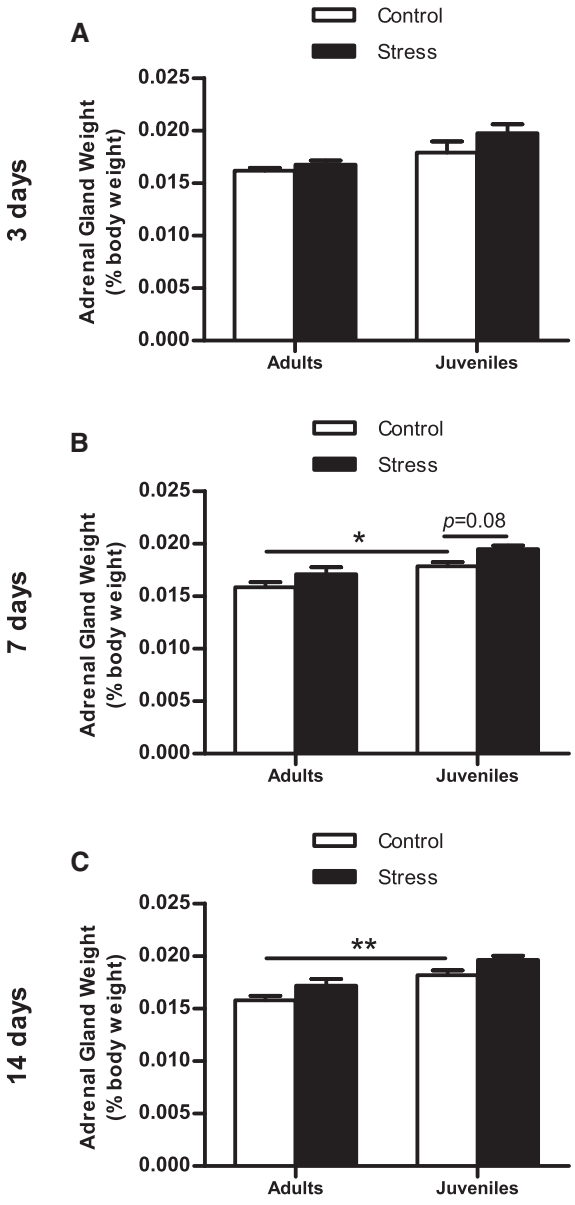

Fig. 3. Effect of 3, 7 or 14 days restraint stress on adrenal gland weight of adult and juvenile BALB/c mice. Adrenal glands were removed and weighed 2 days after restraint. Results are expressed as mean ± SEM, $n = 7$–8/group. $^{*}p < 0.05$, $^{**}p < 0.01$ (2-way ANOVA).

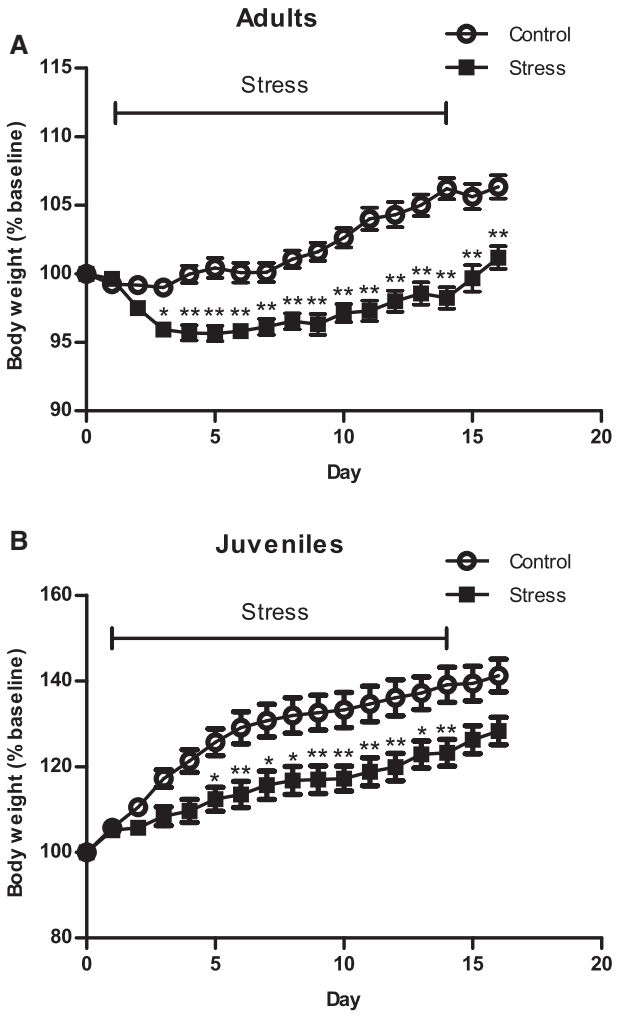

Fig. 4. Effect of 14 days restraint stress on daily bodyweight of adult and juvenile BALB/c mice. Results are expressed as mean ± SEM, $n = 8$/group. $^{*}p < 0.05$, $^{**}p < 0.01$ (repeated measures mixed model analysis).

3.2. Effects of repeated restraint stress on depression-related behaviours

Adult and juvenile BALB/c mice experienced daily restraint stress (2 h per day) for either 3, 7 or 14 days. To investigate the effects of repeated stress, rather than acute effects of restraint stress, behaviour in the FST was assessed 24–48 h following the last restraint (Fig. 5). A significant effect of restraint stress was only observed in adult mice following 3 days of restraint stress. This resulted in a 17% decrease in the time spent immobile, and a 35% increase in the time spent swimming in the FST ($p < 0.05$, *t*-test). Extending the restraint stress for 7 or 14 days in adult mice had no significant effect on either the time spent swimming or immobile (all *p*'s > 0.3). In juvenile mice, behaviours in the FST were not affected by 3 or 7 days of restraint stress (all *p*'s > 0.6). Conversely, increasing the duration of restraint to 14 days resulted in a significant 27% decrease in the time spent immobile, and 37% increase in the time spent swimming in the FST ($p < 0.05$, *t*-test). To determine whether there was any persistent effect of restraint stress into adulthood, juvenile mice who underwent 3 days restraint stress were re-tested in the FST as adults, aged 9–10 weeks old. No significant difference between stressed and control mice was found in either the time spent immobile, or swimming, in the FST (both *p*'s = 0.6, Supplementary Fig. 3A).

The effect of 3, 7 and 14 days restraint stress in BALB/c mice was also assessed in the SPT (Fig. 6). In preliminary experiments, 2.5% sucrose was not sufficient to induce a preference in BALB/c mice whereas a 5% sucrose solution provoked a robust sucrose preference in BALB/c mice (Supplementary Fig. 4). Interestingly, 2.5% sucrose was sufficient to induce a clear preference in both control and stressed C57BL/6 mice (Fig. 10B) reflecting the different sensitivities of inbred mouse strains for sucrose [25]. The effect of both stress and age on sucrose preference was determined for each duration of stress using a two-way ANOVA, with age and stress as factors. There was a significant main effect of stress in 3 days restraint stressed mice ($F_{(1,25)} = 4.58$, $p < 0.05$). While this was associated with a 10% decrease in sucrose preference in both adult and juvenile stressed mice compared with control, this was not statistically significant following pairwise comparisons ($p = 0.2$). There was no significant difference in total consumption of both water and sucrose between stressed and control mice in either adults or juveniles following 3 days restraint (*p*'s > 0.1). Following 7 days stress, there was a significant effect of stress ($F_{(1,27)} = 7.29$, $p < 0.05$), age ($F_{(1,27)} = 7.91$, $p < 0.01$) and age*stress interaction ($F_{(1,27)} = 5.35$, $p < 0.05$) on sucrose preference. In adults, there was a 33% increase in sucrose

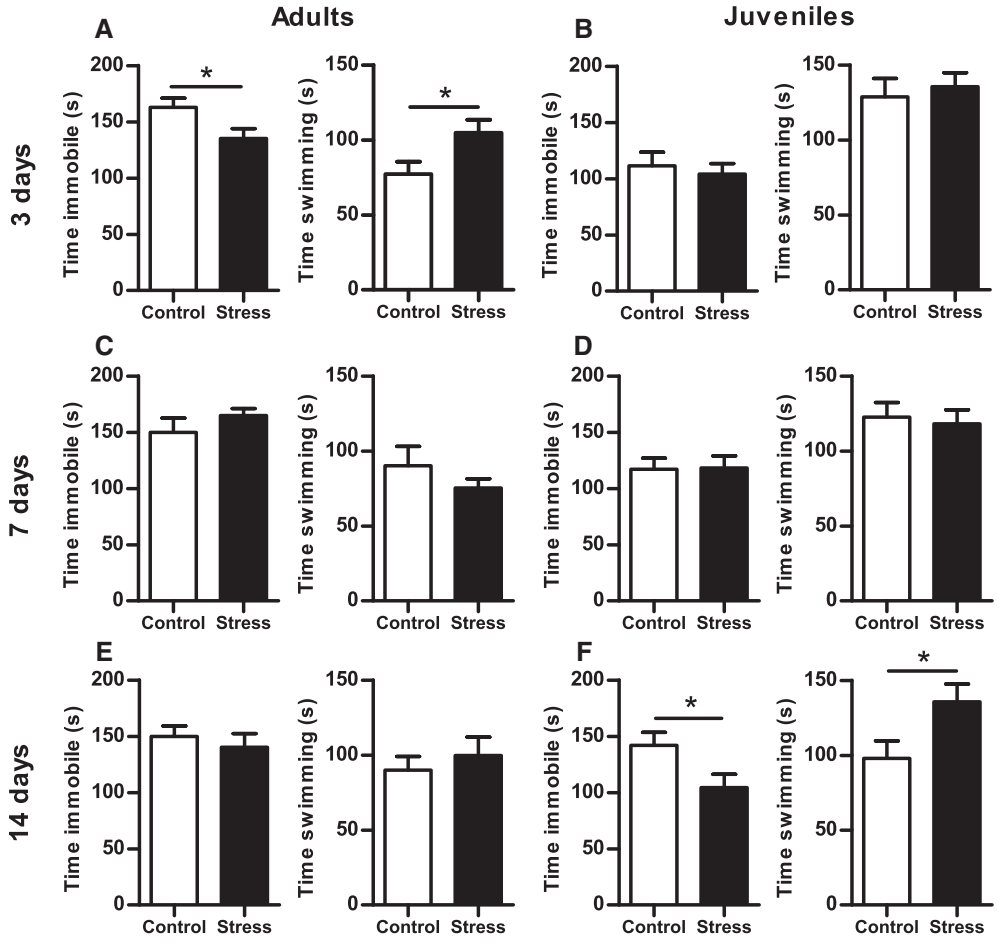

Fig. 5. Effect of 3, 7 or 14 days restraint stress on behaviour in the FST in adult and juvenile BALB/c mice. Time spent swimming and immobile were recorded in the last 4 min of a 6 minute test. Results are expressed as mean ± SEM, $n = 7$–12/group. *$p < 0.05$ compared to control (unpaired t-tests).

preference in stressed mice compared with controls ($p < 0.01$), although the controls in this experiment showed no preference for sucrose so this apparent increase may be anomalous. This could have arisen because we used a "threshold" sucrose concentration of 5% which induces preference in all other control animals, but not in this group of animals. There was no effect of 7 days stress on sucrose preference in juvenile mice. There was no effect of 7 days restraint on total consumption ($F_{(1,27)} = 0.75$, $p = 0.4$). 14 days restraint resulted in a 12% reduction in sucrose preference in adult mice, and a 16% reduction in juveniles ($F_{(1,26)} = 16.59$, $p < 0.001$). This was accompanied by a significant decrease in consumption in juvenile, but not adult, stressed mice compared with controls ($p < 0.05$).

Repeated restraint stress therefore provoked an apparent antidepressant-like effect on behaviours in the FST after 3 days restraint stress in adults or 14 days restraint stress in juveniles. However, in the SPT, the behavioural response to repeated stress manifested as a significant decrease in preference for sucrose in juveniles, and a trend towards a significant decrease in adults, suggestive of an anhedonic or prodepressant response to 14 days restraint stress.

3.3. Effects of repeated restraint stress on anxiety-related behaviours

Separate groups of BALB/c mice underwent a similar restraint stress protocol and behaviour was evaluated in the EPM 24–48 h following the last restraint session (Fig. 7). In adult mice, only 7 days restraint stress resulted in a significant change in behaviour, evident as a 130% increase in the time spent in the open arms in stressed mice compared with control ($p < 0.05$, t-test). Importantly, there was no change in total locomotion in these mice indicating that this was an apparent anxiolytic-like response. However, in juvenile mice, the duration of restraint stress showed a mixed pattern of behavioural changes. After 3 days repeated restraint there was no change in the time spent in the open arms but a 200% increase in the number of open arm entries ($p < 0.01$, t-test) was evident, associated with a 160% increase in locomotor activity ($p < 0.001$, t-test). 7 days restraint resulted in a 220% increase in the time in the open arms ($p < 0.05$, t-test), and a 170% increase in the number of open arm entries ($p < 0.01$, t-test) without a change in locomotor activity. Following 14 days restraint a significant 71% increase in locomotor activity was evident ($p < 0.01$, t-test), although there was no change in either the time spent in, or entries into, the open arms of the EPM (both p's > 0.1, Fig. 7). To determine whether 3 days restraint stress in juvenile mice would manifest as a behavioural change in adulthood, juvenile mice who underwent 3 days restraint stress were re-tested in the EPM as adults, aged 9–10 weeks old (Supplementary Fig. 3). There was no significant difference between stressed and control mice in the time spent in, or the number of entries into the open arms, or total locomotion in the EPM (all p's > 0.1, t-test). There was an apparent increase in open arm entries in the EPM, suggestive that the anxiolytic-like response seen as juvenile mice may persist into adulthood.

To compare the behavioural effects of repeated restraint stress with a single application of restraint, adult and juvenile mice were tested in either the FST, SPT or EPM the day after one 2 h session of restraint (Supplementary Fig. 5). There was no significant effect of acute stress on the time spent either swimming or immobile in the FST ($p = 0.5$), on

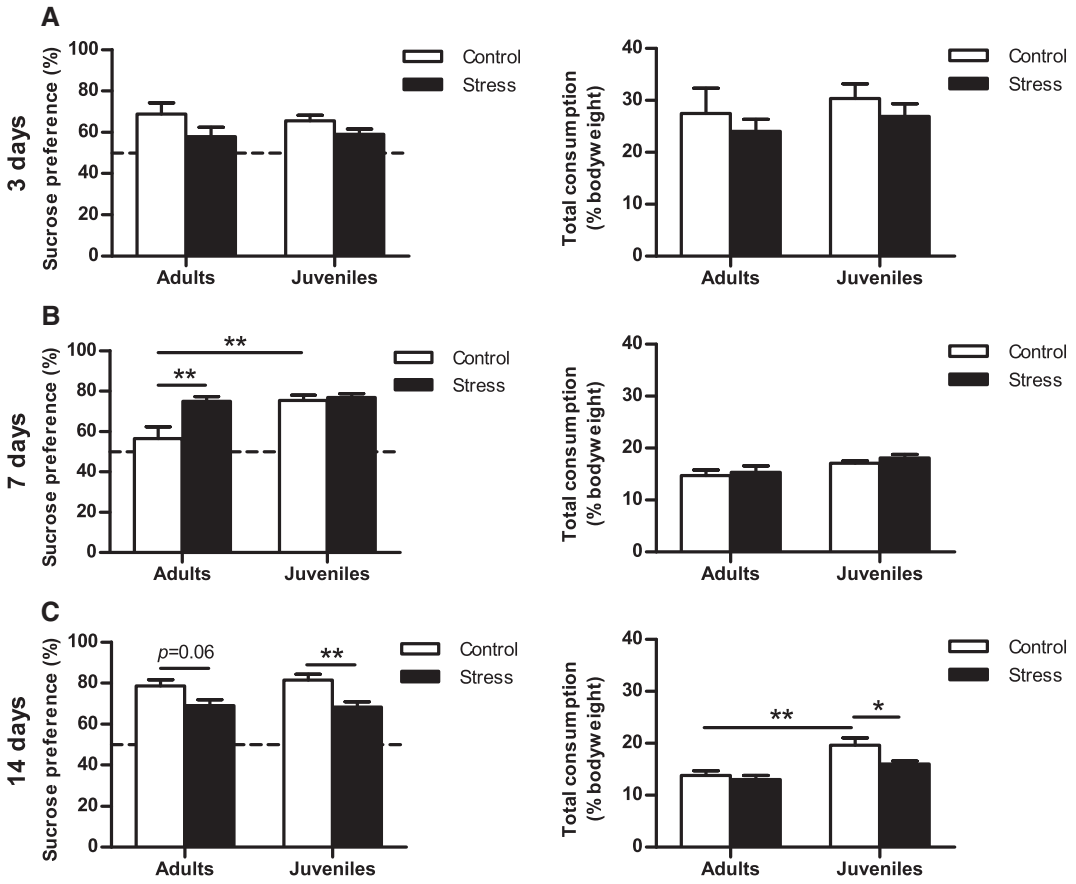

Fig. 6. Effect of 3, 7 or 14 days restraint stress on sucrose preference in adult and juvenile BALB/c mice. Preference for 5% sucrose solution, and total consumption of both sucrose and water were measured over a 12 h test period (7 pm–7 am). Dotted line represents position of no preference. Results are expressed as mean ± SEM, $n = 6$–8/group. $^{**}p < 0.01$ (2-way ANOVA).

sucrose preference ($p = 0.5$), or on the time spent in the open arms, number of open arm entries, or total locomotion in the EPM (all p's > 0.2).

3.4. Effects of repeated restraint stress in C57BL/6 mice

The BALB/c mouse strain has been reported to be relatively stress sensitive whereas the C57BL/6 strain is relatively stress resilient [22]. Therefore we compared the behavioural and neuroendocrinological effects of 3 days restraint stress in C57BL/6 adult and juvenile mice with BALB/c mice. In both adult and juvenile C57BL/6 mice, 3 days restraint stress was associated a 4% decrease in body weight (Adults: $F_{(1,12)} = 15.63$, $p = 0{,}002$; Juveniles: $F_{(1,14)} = 1.97$, $p = 0.18$). As with BALB/c mice, there was a significant effect of stress on corticosterone in both adult ($F_{(1,27)} = 109.79$, $p < 0.001$) and juvenile ($F_{(1,29)} = 190.51$, $p < 0.001$) C57BL/6 mice (Fig. 8). 3 days restraint stress resulted in a 220% increase in corticosterone over baseline in adult mice, and a 310% increase in juvenile mice ($p < 0.001$). This was accompanied by a 9% increase in adrenal gland weight in stressed adult mice, and a 12% increase in juvenile stressed mice, compared with non-stressed controls ($p < 0.01$).

While qualitatively similar, the corticosterone response in stressed BALB/c mice showed a bigger % increase compared to baseline than that seen in C57BL/6 mice. To determine whether repeated restraint stress differentially affected negative feedback of the HPA axis in these two strains, we used the dexamethasone suppression test (Fig. 9). Dexamethasone significantly suppressed corticosterone release, compared with baseline, in both adult and juvenile BALB/c ($F_{(1,28)} = 105.1$, $p < 0.001$) and C57BL/6 mice ($F_{(1,28)} = 64.7$, $p < 0.001$). Stress had no significant effect on dexamethasone-induced suppression of corticosterone release in either strain (both p's > 0.1).

The behavioural effects of 3 days daily repeated restraint stress in C57BL/6 mice were qualitatively similar to those seen in BALB/c mice. In the FST behavioural changes were observed in adult, but not juvenile, C57BL/6 mice evident as a 50% decrease in the time spent immobile, and a 65% increase in the time spent swimming, after 3 days of restraint stress ($p < 0.01$, Fig. 10). In the SPT, a two-way ANOVA revealed a significant effect of age ($F_{(1,26)} = 4.35$, $p < 0.05$) on sucrose preference, but no significant effect of restraint stress ($F_{(1,26)} = 2.79$, $p = 0.1$) or stress * age interaction ($F_{(1,26)} = 1.58$, $p = 0.2$). Interestingly, there was also a significant effect of age ($F_{(1,26)} = 41.9$, $p < 0.0001$) and stress ($F_{(1,26)} = 7.08$. $p = 0.01$) on total sucrose consumption. Juvenile mice drank significantly more than adult mice for both stressed and control animals ($p < 0.001$). In the EPM, in juvenile mice restraint stress resulted in a 100% increase in the time spent in the open arms ($p < 0.01$), without a change in total locomotion, consistent with an anxiolytic-like effect. Similarly, in adult mice, there was a 45% increase in time spent in the open arms following 3 days restraint, which had a trend towards statistical significance ($p = 0.07$, Fig. 10).

4. Discussion

We have shown for the first time the behavioural and hormonal effects of repeated restraint stress in both adult and juvenile BALB/c mice and C57BL/6 mice. Repeated restraint stress of different durations (3, 7 and 14 days) provoked a robust physiological response in BALB/c mice. Plasma cortisol levels increased and body weight decreased in stressed, compared to control non-stressed, adult and juvenile mice. Interestingly, adrenal gland weight, expressed as a percentage of body weight, was higher in juvenile mice than in adult mice, and showed a trend to increase in stressed, compared to control, adult and juvenile mice. Increases in corticosterone following 3 days stress appeared to be

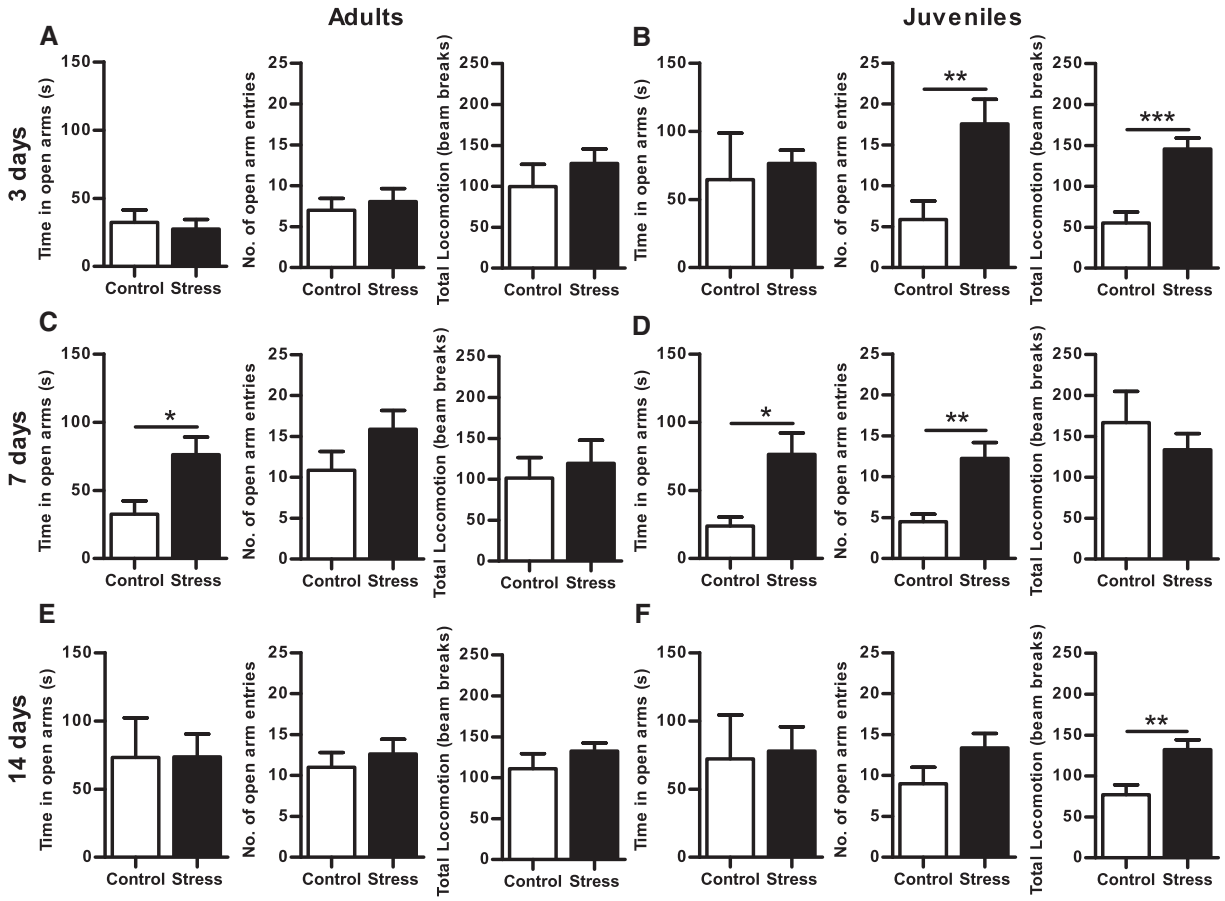

Fig. 7. Effect of 3, 7 or 14 days restraint stress on behaviour in the EPM in adult and juvenile BALB/c mice. Time spent in the open arms, number of entries into the open arms and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test period. Results are expressed as mean ± SEM, $n = 7$–12/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired *t*-tests).

greater in juvenile mice than in adults, in both strains of mice studied, suggestive of the increased sensitivity of the HPA axis seen in juvenile animals [29,30]. Previous studies have observed that restraint stress in mice induced increases in corticosterone levels similar to those found in the present study. For example, Romeo et al. showed a significant increase in corticosterone levels, in trunk blood samples from both 30 day old and adult BALB/c and C57BL/6 mice, immediately following a single exposure (30 min) to restraint stress [31]. Corticosterone levels reported were much higher (~700–1200 ng/ml) than the values we obtained following 3 days repeated restraint stress in blood samples collected via the tail nick method [26]. Romeo et al. also showed that restraint stress in juvenile BALB/c mice induced a higher corticosterone response than in adults [31]. However, our data shows a clear attenuation of the corticosterone response in both adult and juvenile BALB/c mice with increasing duration of restraint stress. Corticosterone was no longer significantly increased above baseline in adult mice following 14 days restraint, indicating habituation to the stressor. This is consistent with previous reports in rats showing that the HPA axis is less responsive to repeated restraint stress following either 8 or 14 days restraint, compared to an acute exposure to the stressor [32,33].

The behavioural changes seen in response to repeated restraint stress were complex. In the EPM, in juvenile mice, 3, 7 and 14 days of repeated restraint stress produced behavioural changes that included an *increase* in the number of open arm entries and/or time spent in the open arms, with and without changes in locomotor activity. This effect on exploratory behaviour in the EPM was not observed in adult mice. Similar anxiolytic-like effects of repeated restraint stress were also evident in juvenile but not adult C57BL/6 mice after 3 days of exposure to the stressor. Interestingly, there was little effect of restraint stress on behaviours in the FST for BALB/c mice, where only the groups of adult mice with 3 days restraint stress, and juvenile mice with 14 days restraint stress, showed a *decrease* in time spent immobile, consistent with an antidepressant-like response. The same 3 day treatment in adult C57BL/6 mice also produced an antidepressant-like effect in the FST. In the SPT, a change in hedonic motivation was evident in both adult and juvenile BALB/c mice. Only after 14 days of repeated stress was a significant decrease in preference for sucrose observed, consistent with a pro-depressant phenotype. The differing nature of the stress-induced behavioural changes in the SPT, compared with the FST and EPM, likely reflects the nature of each test. Both the FST and EPM assess a behavioural response to a stressful environment, whereas the SPT measures preference for a palatable substance in the home cage, a non-stressful environment.

One interpretation of these data is that the repeated restraint stress leads to a behavioural response that is an adaptive biological process. As a result of prior stress exposure, mice may adapt their behaviour as a stress-coping strategy. This behavioural change may be beneficial in future stress challenges. Thus, after repeated restraint stress, behaviour in the EPM manifests as an apparent anxiolytic-like response or behaviour in the FST manifests as an antidepressant-like response. Resilience has been suggested to be either an active, adaptive response, resulting in antidepressant and anxiolytic-like changes in behaviour [34,35], or a more passive mechanism, manifesting in an absence of pathological changes following chronic stress [34]. The results shown here may reflect a mixture of both active and passive mechanisms of resilience, as shown by both an increased exploration of the open arms of the EPM following restraint stress in some cohorts of mice, as well as an absence of depression- or anxiety-related behaviours in others. The predictable nature

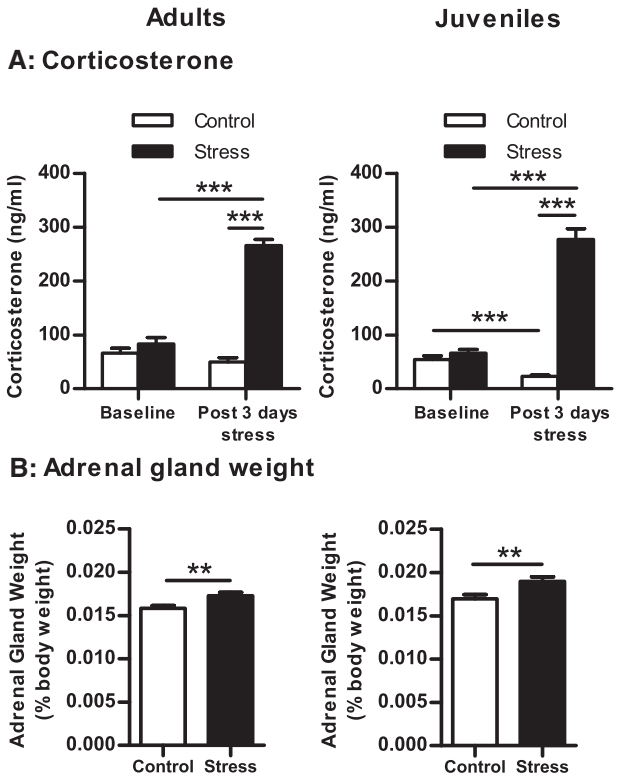

Fig. 8. Effect of 3 days restraint stress on plasma corticosterone (A) and adrenal gland weight (B) in adult and juvenile C57BL/6 mice. (A) Blood samples were taken at baseline and immediately following 3 days restraint stress. (B) Adrenal glands were removed and weighed the day after the last session of restraint. Results are expressed as mean ± SEM, $n = 15–21$/group. $^{**}p < 0.01$, $^{***}p < 0.001$, repeated measures mixed model analysis (A), unpaired *t*-tests (B).

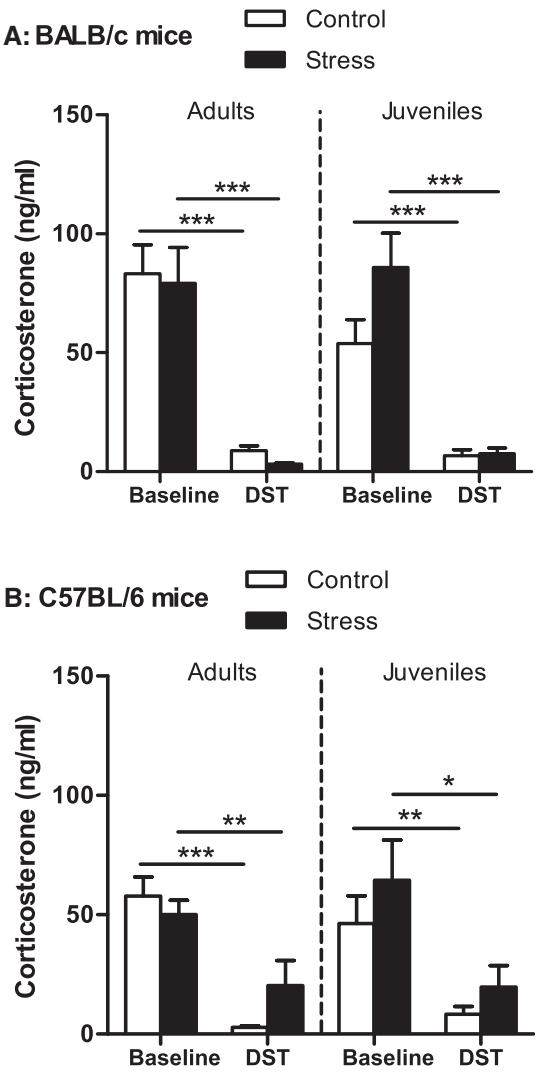

Fig. 9. Effect of 3 days restraint on the dexamethasone suppression test in adult and juvenile BALB/c (A) and C57BL/6 (B) mice. Blood samples were taken at baseline and 6 h following administration of dexamethasone (0.1 mg/kg, i.p.). Results are expressed as mean ± SEM, $n = 8$/group. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (repeated measures mixed model analysis).

of the restraint stress protocol used here may make it comparatively less stressful than unpredictable stressors [36]. This in turn could also contribute to the development of resilience rather than an anxious or depressive phenotype and may make this paradigm a useful approach to studying the molecular mechanism underlying resilience.

In considering whether the behavioural changes are an active or passive adaptation to stress, the increase in locomotor activity in the EPM may be an important factor. Rather than a typical passive avoidance of the aversive open arms of the EPM, increased locomotor activity may reflect an increase in a drive to escape the aversive environment [37]. Increased locomotion in an open field has also been seen following restraint stress and this stress-induced hyperlocomotion has been used as a marker of stress-responsivity in mice [38]. An increase in mobility in the FST and the similar tail suspension test have also been reported following chronic stress, again suggesting an active defence mechanism in response to the aversive nature of the behavioural tests [39]. The increased locomotion seen here in the EPM and the reduction in immobility in the FST following stress, may be reflective of an active response to stress, rather than being interpreted as a reduction in depression- or anxiety-related behaviours.

Direct comparisons of this study with other reports in the literature are difficult because of differences in restraint stress procedures. It is known that the effects of restraint stress depend on the duration, frequency and intensity of restraint [16]. Acute restraint stress, a single application, is most commonly used in the literature with rats being studied more often than mice. In comparing restraint paradigms used in adolescent mice a variety of devices have been used; including a modified 50 ml syringe tube as we have used [17,40], a plastic restraint bag [41,42] or a wire mesh cage [31,43]. How control animals are handled also varies across laboratories where non-stressed animals may not be handled at all [44] or perhaps removed to a novel environment [40]. In our study the control non-stressed animals were gently handled and weighed daily to ascertain that any behavioural or hormonal effects are due to the restraint stress itself, rather than the handling which is itself a potential stressor [23]. This may account for changes in control groups observed in some studies, particularly in the longer duration studies where mice will have been handled for up to 14 successive days. The duration of restraint stress also varies across reports in the literature with some mouse studies using single or repeated restraint stress sessions of 15 min [42], 30 min [31], 3 h [40], 8 h [43] or longer [17]. Behavioural assessments typically are conducted 30–60 min post restraint stress in a variety of paradigms. In this study, we have evaluated behaviour in a variety of tests including the elevated plus maze, the forced swim test and the sucrose preference test 24–48 h post-restraint to assess whether there was lasting impact on the behaviour of the animal from the repeated restraint stress sessions. For example, it has been shown that acute restraint stress in mice does induce anxiety- and depression-related behaviours in the EPM and FST [45,46]. In our experiments (Supplementary Fig. 5), 24 h following an acute restraint stress, no significant changes in behaviour were

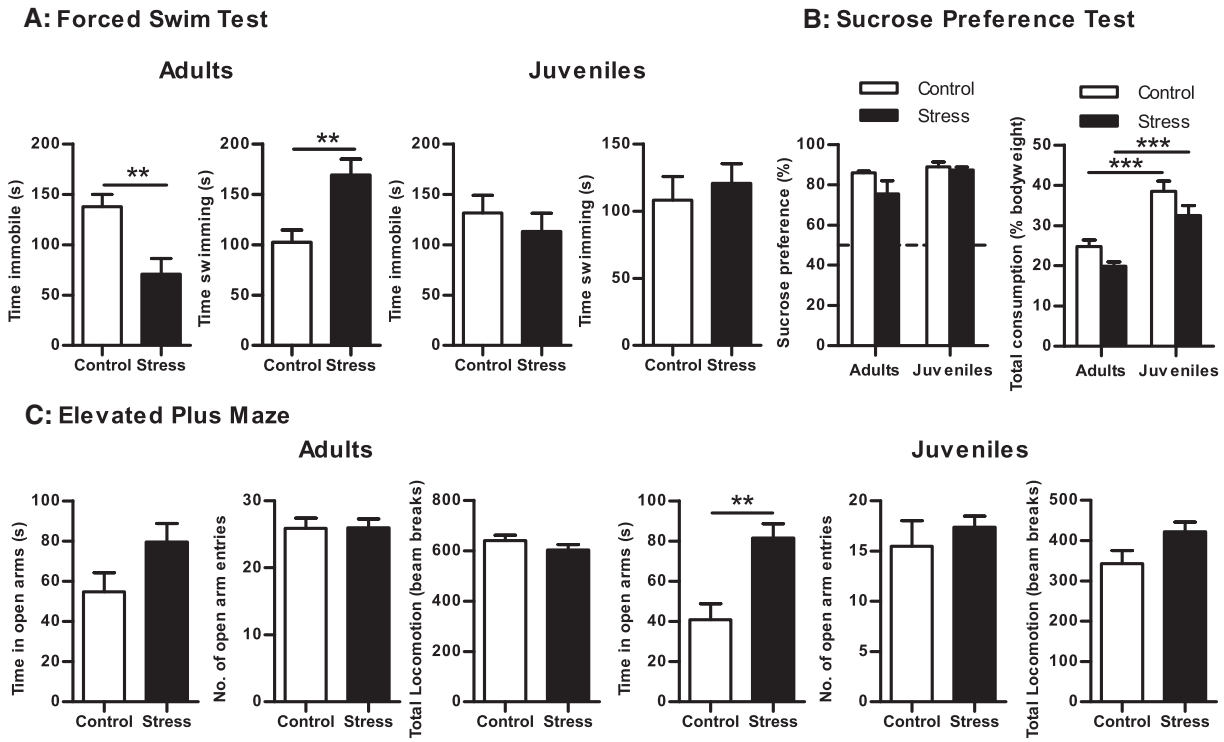

Fig. 10. Effect of 3 days restraint on behaviour in the forced swim test (A), sucrose preference test (B) and elevated plus maze (C) in adult and juvenile C57BL/6 mice. (A) Time spent immobile and swimming was measured in the last 4 min of a 6 minute test. (B) Preference for 2.5% sucrose solution, and total consumption of both sucrose and water were measured over a 12 h test period (7 pm–7 am). Dotted line represents position of no preference. (C) Time in the open arms, number of open arm entries and total locomotion over the whole maze were assessed by beam breaks in a 5 minute test session. Results are expressed as mean ± SEM, n = 8–12/group. $^{**}p < 0.01$, unpaired t-tests (A, C), 2-way ANOVA (B).

seen in the FST, SPT or EPM. This timing of behavioural assessment is important if we are to distinguish between the acute effects of restraint stress and the long-lasting chronic effects of restraint. This appears to be a novel feature of our experimental design and therefore comparable data in adult and juvenile mice are limited. A final consideration is the blood sampling methodology we have used. Different laboratories report the impact of the restraint stress on corticosterone levels from blood samples either immediately after restraint stress or 15–60 min post-restraint and typically using post mortem trunk blood samples. Romeo et al. have shown the dynamics of the corticosterone response in adolescent mice following a single 30 min restraint session [31]. Corticosterone levels are highest immediately post-stress but return close to baseline levels 1 h post-stress. In this study, blood sampling was performed at baseline and immediately following the last restraint session. We have adapted a tail incision method for repeated sampling from the lateral tail vein in juvenile mice that we have shown previously to be non-stressful [26,47]. Thus while our experimental design may preclude the observation of acute behavioural effects following a single restraint stress session (Supplementary Fig. 5); it does show that the behavioural responses we have observed persist (24-48 h) after repeated restraint stress and represent an adaptive response.

In this study we have compared the effects of restraint stress on two different strains of mice. There have been several studies looking at strain differences in stress responsiveness, with BALB/c mice considered to be more sensitive to the effects of stress than the more stress-resilient C57BL/6 strain [22,36]. Consistent with this, our results show an increased corticosterone response to 3 days restraint stress in BALB/c mice (700% increase above baseline) compared with C57BL/6 mice (220% increase above baseline). In the forced swim test, 3 days restraint stress resulted in antidepressant-like reductions in immobility in both BALB/c and C57BL/6 adult mice, and this appeared more pronounced in C57BL/6 mice (50% decrease from control) than in the BALB/c strain (20% decrease from control). Again, this suggests increased resilience in C57BL/6 mice, compared to the more stress sensitive BALB/c strain. BALB/c mice used throughout these studies were housed individually to avoid fighting, whereas the less aggressive C57BL/6 mice were housed in groups. While the effect of housing conditions in the present study is unknown, it has previously been shown that individual housing of male BALB/c and C57BL/6 mice had no effect on either corticosterone levels or anxiety-related behaviour [48], suggesting the effect of housing conditions may be limited.

In humans it has been shown that exposure to stress, particularly during childhood and adolescence, promotes the development of resilience [49,50]. In addition, heightened responses to stress reactivity in adolescents, compared with adults, have been shown in both mice and humans [51]. We have proposed here that when juvenile mice are stressed and tested as juveniles, they respond with an adaptive behaviour that may represent a stress coping mechanism. When we examined whether stress-induced effects in juvenile animals persisted into adulthood (Supplementary Fig. 3), there was an apparent increase in open arm entries into the EPM. This data is consistent with the idea that juvenile mice show a greater anxiolytic-like response to stress than adult mice do which may persist into adulthood. This is consistent with previous reports that social crowding stress is anxiolytic in juvenile mice, but not in adults [52]. Heightened responses to stress reactivity in adolescents, compared with adults, have also been shown in both mice and humans [51]. The juvenile mice used in this study were aged 4–6 weeks during exposure to stress and this period corresponds to adolescence in humans [5]. It is known that brain areas such as the hippocampus, hypothalamus, prefrontal cortex and amygdala, key regions involved in the stress response and regulation of emotion and behaviour, continue to develop throughout adolescence and into young adulthood [5]. Together, our findings support the idea that while adolescence may be a particularly sensitive period for the effects of stress exposure

[4,5,30]; repeated exposure to a predictable stressor may promote resilience and adaptive stress coping behaviours.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.physbeh.2016.09.014.

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Validation of a refined technique for taking repeated blood samples from juvenile and adult mice

Annelisa M Sadler and Sarah J Bailey

Abstract

Repeated blood sampling from laboratory animals is desirable in certain experimental designs and also for reducing the number of animals used in research. Biochemical methods for analysing blood samples require only small blood volumes to be collected (typically 20–40 μL). In juvenile mice, the small blood volume of the animals also requires only small samples to be taken. Furthermore, for behavioural studies it is desirable to have a method that does not require anaesthesia or the use of invasive indwelling cannulae. We report the validation of a refined method for repeated blood sampling (up to 3 times at 24 h intervals) in juvenile and adult mice using the tail incision method to sample from the lateral tail vein. This method is not stressful, as assessed by low basal levels of the stress hormone corticosterone. Since repeated blood samples can be collected from the same animal at multiple time points, it is not necessary to increase group size for terminal sample collection. Thus, in addition to being a refined method requiring no warming of the tail, no anaesthesia and only gentle restraint, this method also reduces the numbers of mice used for experiments.

Keywords

rodents, stress, bleeding techniques, corticosterone, tail vein

Numerous techniques exist for the collection of blood from mice.[1] While taking blood samples from mice is a common practice in the laboratory setting, the procedure can be stressful due to the handling, restraint or anaesthesia involved in the procedure.[1,2] Taking repeated blood samples from the same mouse allows a significant reduction in the number of mice used over the course of an experiment. Hence a refined minimally invasive method of blood sampling, which allows multiple samples to be taken from the same animal, is desirable.[3] However, in the UK, Home Office guidelines limit repeated sampling and indicate that no more than 10% of the total blood volume from a mouse may be taken on each occasion, with no more than 25% being taken over a 28-day period.[2,4]

In our research we are interested in studying the behavioural effects of stress, over time, in juvenile mice and assessing the impact of stress via measurements of neuroendocrine function, e.g. corticosterone analysis. This presents several challenges when considering repeated blood sampling methodology. Firstly, the stressful nature of the blood sampling technique itself may confound the results of corticosterone analyses. Secondly, when working with juvenile mice (3–6 weeks old), which weigh as little as 12 g, this limits the volume that can be taken on each occasion to less than 60 μL. The lateral tail vein is an appropriate route for repeated sampling of small blood volumes from mice without anaesthesia, although vasodilation may be required.[2,4] We compared three different blood collection techniques in adult mice before adapting and validating the method developed by Fluttert et al.[5] as a refined method for repeated blood sampling in juvenile mice.

Male BALB/cAnNCrl mice (Charles River, Margate, UK), aged 3–4 weeks old (juvenile) or 7–8 weeks old (adult), were housed individually in 35 × 20 × 15 cm polysulfone cages (Plexx, Elst, The Netherlands) with woodchip bedding (Datesand, Manchester UK) and paper nesting material (Lillico/

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LBS Biotechnology, Horley, UK). The mice were maintained in a temperature ($21 \pm 1^{\circ}C$) and humidity (50–60%) controlled environment, under a 12 h light/dark cycle (lights on 07:00 h), with food and water available ad libitum. All the mice were allowed to acclimatize to the animal facility for at least one week prior to blood sampling. During this time the animals were handled by gentle cupping[6] for 2–3 min on 2–3 days prior to blood sampling. All procedures were carried out under a Home Office project licence held in accordance with the Animals (Scientific Procedures) Act 1986.

Adult mice had a blood sample taken from the lateral tail vein by one of three methods ($n = 5$ per treatment group). All the samples were collected in heparinized capillary tubes (Fisher Scientific, Loughborough, UK) between 11:00 h and 13:00 h. The first method involved warming the mice in a thermostatically controlled warming chamber, at 38°C for 15 min in line with guidelines on the NC3Rs microsampling website.[2] The mice were then briefly restrained in a restraint device, a 25 G needle was inserted into the lateral tail vein, and resulting blood droplets were collected. The second method involved holding the mice by hand, and immersing the tail in warm water at 42°C for 20 s. A 25 G needle was again inserted into the lateral tail vein, and resulting blood droplets were collected. The third method was the tail incision method[5] which involved one person gently cupping the mice by hand,[6] while the operator held the tail gently on the bench and made a small nick (approximately 2 mm wide × 0.5 mm deep) in the tail with a razor blade, perpendicular to the tail vein, approximately 2 cm from the tip of the tail. Blood droplets were directly collected into capillary tubes. In the tail incision method blood flow was encouraged by gently stroking the tail and in the majority of cases, blood flow stopped spontaneously when stroking was stopped. On occasion it was necessary to apply a small amount of pressure to the tail to stop bleeding. In all cases, the blood samples were immediately transferred to microcentrifuge tubes containing EDTA (final concentration in sample 3 μg/μL), and stored on ice. Twenty-four hours after the blood sample was taken, all the mice were culled by cervical dislocation, rapidly decapitated and a second, terminal blood sample was collected directly into microcentrifuge tubes containing EDTA (final concentration in sample 3 μg/μL). All the tubes were centrifuged at 2000 relative centrigual force (rcf) for 20 min at 4°C. Plasma was removed and stored at −20°C until analysis. The concentration of corticosterone in each plasma sample was determined using a corticosterone enzyme-linked immunosorbent assay (ELISA) (IBL International, Hamburg, Germany). Plasma was diluted 1:10, and the ELISA was carried out according to the manufacturer's instructions.

In adult mice (Figure 1), two-way repeated measures mixed model analysis[7] revealed a significant main effect of sampling method ($F_{(2,12)} = 7.08$, $P < 0.01$), baseline or terminal sample ($F_{(1,12)} = 21.16$, $P < 0.001$) and a significant method*sample interaction ($F_{(2,12)} = 10.78$, $P < 0.005$). Post hoc analysis revealed that corticosterone was significantly higher in samples taken by the warming cabinet method compared with both the tail warming method (330 ± 58 ng/mL versus 110 ± 13 ng/mL, $P < 0.005$) and the tail incision method (330 ± 58 ng/mL versus 74 ± 13 ng/mL, $P < 0.001$), clearly indicating that the warming cabinet was stressful (Figure 1). There was no significant difference between the tail warming and tail incision methods (110 ± 13 ng/mL versus 74 ± 13 ng/mL, $P = 0.18$), or between the tail warming/incision methods and their respective terminal samples (Figure 1).

We then went on to develop the tail incision method for repeated sampling in juvenile mice ($n = 4/5$ per treatment group). Blood samples were taken from juvenile mice (1) at baseline, (2) 24 h later following a 2 h restraint stress and (3) 24 h post-stress. A tail incision was made starting approximately 2 cm from the tip

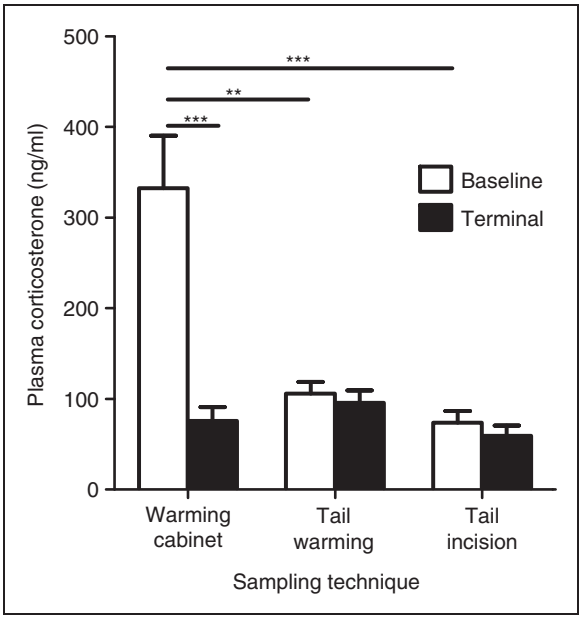

Figure 1. Effect of three different blood sampling methods on plasma corticosterone levels in adult (7–8 weeks old) BALB/cAnNCrl male mice. 'Baseline' samples were obtained from the lateral tail vein following either warming in a cabinet, or tail warming by immersion in water or tail incision method (no warming). Animals were killed 24 h later by cervical dislocation and 'terminal' samples were obtained. Results are expressed as mean ± SEM, $n = 5$/group. $^{**}P < 0.005$, $^{***}P < 0.001$ (two-way repeated measures mixed model analysis).

of the tail, and on repeated sampling a different location was used, working towards the base of the tail in 0.5 cm increments. Corticosterone was significantly increased by 2 h of restraint stress compared with baseline (720 ± 59 ng/mL versus 21 ± 9.8 ng/mL), and this increase had returned to baseline within 24 h following stress (24 ± 3.4 ng/mL versus 720 ± 59 ng/mL, Figure 2a). We then confirmed that heating and restraining mice to take a blood sample was stressful in juvenile mice in the same way as in adults. Mice had a blood sample taken at baseline by the warming cabinet method. Twenty-four hours later they were restrained for 2 h and a second blood sample was taken in the same way. The results confirmed our previous findings that this method of taking blood is stressful (Figure 2b).

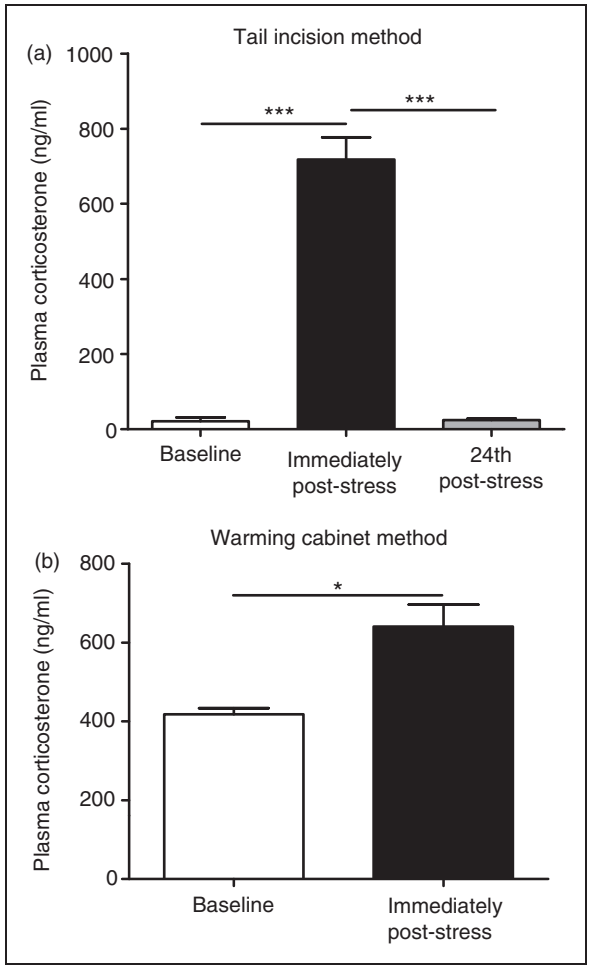

Figure 2. Effect of stress on plasma corticosterone in juvenile (4–5 weeks old) male BALB/cAnNCrl mice following repeated blood collection. (a) Blood samples were taken on three successive days using the tail incision method at baseline (day 1), immediately following stress (day 2) and 24 h post-stress (day 3). (b) Blood samples were taken by the warming cabinet method at baseline (day 1) and immediately following stress (day 2). Results are expressed as mean ± SEM, $n = 4$–5/group. $^{*}P < 0.05$, $^{***}P < 0.001$ (two-way repeated measures mixed model analysis (a), paired *t*-test (b)).

Here, we have demonstrated that the tail incision method is a refined method of taking repeated blood samples from juvenile mice. The low basal levels of the stress hormone corticosterone obtained using the tail incision method compared with the warming cabinet method indicate that this is not a stressful procedure. Furthermore, the repeated nature of sampling is not stressful as the third sample taken using the tail incision method did not increase plasma corticosterone levels above the baseline sample on the first day of sampling (Figure 2a). This method was originally developed for use in adult rats,[5] particularly for use in behavioural studies where implantation of indwelling cannulae is not desirable or methods like tail-cuts are unsuitable for repeated sampling. Our data demonstrate that this methodology is applicable to juvenile mice. Furthermore, we have shown that in both adult and juvenile mice, the use of a warming chamber is stressful, producing a significant ~3-fold increase in plasma corticosterone levels. The stressful nature of these techniques therefore precludes their use in studies examining effects of stress. An additional benefit of the tail incision method is that it allows blood flow to be started and stopped easily, facilitating the collection of very small volumes of blood (typically 20–40 µL) from juvenile mice. Pilot studies in our laboratory involving the use of the saphenous vein were not successful in this respect. Finally, the ability to take repeated samples from the same mouse at multiple time points reduces the number of animals required for an experiment, compared with the taking of terminal blood samples from large numbers of mice, and is thus in keeping with the principles of both reduction and refinement of animal use in laboratory research.[3]

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